BONE MORPHOGENETIC PROTEIN-2 IN THE TREATMENT AND DIAGNOSIS OF CANCER

PRIORITY CLAIM

[0001]   The present application is a continuation-in-part of US App. No. 10/044,716, filed January 11, 2002, which is a converted utility application claiming the benefit of U.S. Provisional Application No. 60/261,252 (Langenfeld), filed January 12, 2001, all of which are incorporated by reference in their entirety herein.

FIELD OF USE

[0002]   The present invention relates to the fields of molecular biology, immunology, and medicine and provides methods for the treatment and diagnosis of cancer. Specifically, it relates to the use of bone morphogenetic protein-2 (BMP-2), as a target for tumor and cancer treatment therapies, a marker to diagnose cancer, and as using antagonists to BMP-2 to reduce neoangiogenesis and continued vascularization of tumors.

BACKGROUND OF THE INVENTION

[0003]   Various publications or patents are referred to in parentheses throughout this application. Each of these publications or patents is incorporated by reference herein. Complete citations of scientific publications are set forth in the text or at the end of the specification.

[0004]   Lung cancer is the leading cause of cancer deaths in the United States with an estimated 157,000 people expected to die from the disease in 2003. (1) Despite

improvements in diagnosis and treatment, only 15% of lung cancer patients survive five years (1) with the majority of patients succumbing due to spread of the cancer to other parts of the body. The genes that induce the invasion and metastasis of lung cancers are still poorly understood.

[0005]   The bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF) superfamily, which are a phytogenetically conserved group of proteins (2). There are 20 isotypes of the BMPs with BMP-2 and BMP-4 sharing 92% homology (3). BMP-2 and BMP-4 are secreted proteins that induce pluripotential mesenchymal differentiation (4, 5, 6) and are required for the normal embryonic development of many organs including lung and bone (7, 8). Animals with functional knockout of BMP-2/4 die between 6.5 and 9.5 days post conception with little to no mesoderm differentiation.

[0006]   BMP-2 is a powerful morphogenetic protein that has been studied for its ability to induce the cascade of endochondral bone formation. BMP-2 can induce the entire developmental program of endochondral osteogenesis when introduced at an ectopic site (9). BMP-2 and BMP-4 also have chemotactic properties capable of inducing the migration of normal vascular endothelial and mononuclear cells (10, 11).

[0007]   The BMPs are synthesized as inactive variable length precursor proteins (12, 13). The precursor BMP-2 proteins are proteolytically cleaved, producing a mature C-terminal 14-kDa protein that is the active peptide (9, 12). Mature BMP-2 protein signaling is mediated by transmembrane serine/threonine kinases called type IA, IB, and type II receptors (14-17). The receptor phosphorylates cytoplasmic targets, which includes the

Smad family of proteins (18). Smads are a class of proteins that function as intracellular signaling effectors for the TGF-β superfamily of secreted polypeptides. The activated BMP type I receptor then phosphorylates Smad1, Smad 5, and/or Smad 8, inducing its translocation into the nucleus and activating the transcription of target genes.

[0008]   While BMP-2 expression has been noted in a few cancers, such as sarcomas (19), pancreatic cancer (20), and in cancer cell lines (21), inhibition of BMP-2 activity as a potential cancer treatment has neither been mentioned nor studied in the literature. To the contrary, several articles suggest that BMP-2 has an inhibitory effect on cancer cell proliferation and may be a useful therapeutic agent to treat cancer. (22, 23, 24). Thus, any teaching that BMP-2 is a compound expected to treat cancer or treat the risk of cancer would be new and unexpected.

[0009]   The development of a blood supply is essential for bone formation. BMPs were thought to promote angiogenesis indirectly in developing bone by inducing the expression of VEGF from osteoblasts. BMP-6 was shown to stimulate the migration and tube formation of bovine aortic endothelial cells (BAEC) (27). Functional knockouts of TGF superfamily members demonstrate their role in vasculogenesis. Mice with a functional knockout of Smad 1 or Smad 5 die at approximately 9.5 to 10.5 weeks and have defects in angiogenesis. Smad 5 mutant embryos had enlarged blood vessels, a decrease in smooth muscle cells, and contained mesenchymal cells, which were unable to direct angiogenesis. Mice lacking TGF receptors died in mid-gestation with defects in angiogenesis. However, the BMPs have never been shown to be associated with the

formation of a neovasculature in tumors. This discovery would elucidate a significant relationship.

[0010]   Id has an important role in mediating an angiogenic response. Mice with a double knockout of Id1-Id3 display vascular abnormalities in the forebrain. Id1+/- Id3 -/- mutant mice failed to support the growth of xenograft tumors, which is thought to occur from the inability to form a neovasculature. Sustained expression of Id cells delays the onset of senescence in human endothelial cells. Id has also been shown to stimulate proliferation of epithelial cells. It was recently shown that Id1 mediates BMP-6 induced migration and possibly also mediates tube formation. (27)

[0011]   VEGF is the most potent angiogenic factor and is thought to be essential for tumor growth and metastasis. (29, 30) Transgenic mice studies have confirmed that overexpression of sonic hedgehog can cause tissue-targeted cancer. (31)

[0012]   Noggin, a natural inhibitor of BMP-2, is a secreted protein that binds BMP-2 and BMP-4, thereby preventing their activation of the BMP receptors. (8, 32, 33, 34, 35) Mouse and human noggin are 98% homologous.

[0013]   The inactivation of Rb by either a point mutation or phosphorylation is thought to occur in 100% of lung carcinomas. When Rb is in a hypophosphorylated state, it is able to block the progression through G1 of the cell cycle. When Rb is inactivated by phosphorylation, this allows progression through G1. Cyclin E is required for progression through G1 of the cell cycle. The expression of cyclin E increases during G1 of the cell cycle. Cyclin E then binds to cyclin dependent kinase 2 (ckd-2) leading to Rb phosphorylation. Cyclin E is frequently over-expressed in lung and other cancers and is

thought to contribute to a worse prognosis. Dysregulation of the Ras/Mek/Erk occurs in lung and many other carcinomas. Over-expression of the Ras/Mek/Erk pathway enhances cellular proliferation and promotes transformation. BMP-2 was previously shown to induce Ras/Mek/Erk signaling in osteoblasts.

[0014]   BMP-2/4 has been shown to induce expression of Id1 in breast carcinoma cell lines, endothelial cells, osteoblasts (45), immortalized human stromal (46), and mouse embryonic stem cells (47). Id1-3 are helix-loop-helix proteins that serve as negative regulators of basic helix-loop-helix transcription factors. Id1 is thought to have an important role in tumorigenesis (48-49). Id1 has been shown to immortalize human keratinocytes through activation of telomerase and Rb inactivation (28). Id1 +/- Id3 -/- knockout mice are unable to support xenograft tumor growth, which thought to occur because lack of neoangiogenesis. Id1 and Id2 have been shown to be critical for the progression of G1 of the cell cycle, which occurs at least in part, by inactivation p16, which leads hyperphosphorylation of Rb.

SUMMARY OF THE INVENTION

[0015]   The present invention is related to the discovery that bone morphogenetic protein-2 (BMP-2) is overexpressed in many common human cancers and is linked to cancer invasion and growth. Further, inhibiting BMP-2 activity reduces the size of cancerous tumors in nude mice and down regulates the expression of VEGF and sonic hedgehog in lung cancer cell lines. Thus, the present invention pertains to the use of BMP-2 as a target for cancer treatment therapies and a means to diagnose cancer.

Specifically, the therapeutic component of this invention involves administering to a patient a composition that inhibits bone morphogenetic protein-2 activity. The diagnostic component of the invention involves measuring the BMP-2 level in biological samples from both a patient and a non-cancerous subject and comparing those levels. Elevated levels indicate increased probability of cancer in the patient.

[0016] A primary aspect of the present invention is to provide a method for the treatment of cancer by administering to a patient a therapeutically effective amount of a BMP-2 activity inhibitor. Some cancers that may be treated by this method are carcinomas, including, but not limited to, lung cancer, bladder cancer, breast cancer, colon cancer, kidney cancer, lung cancer, ovarian cancer, thyroid cancer, endometrial cancer, omental cancer, testicular cancer, and liver cancer. In a preferred embodiment of this invention, the patient is human.

[0017] The BMP-2 inhibitor of this invention may be a polypeptide that binds specifically to bone morphogenetic protein-2 itself, a polypeptide that binds specifically to a BMP-2 receptor, or an antibody that binds specifically to BMP-2 or a portion of BMP-2. The BMP-2 inhibitor may also be an antisense oligonucleotide that binds to a BMP-2 nucleic acid sequence or some portion thereof.

[0018] This invention features several particular polypeptides that are BMP-2 inhibitors. Preferred embodiments of this invention feature known antagonists to BMP-2, such as noggin, chordin, cerberus 1 homolog, gremlin, and DAN (2-deoxy-2,3-didehydro-D-N-acetylneuraminic acid). Noggin is particularly preferred. Another aspect of this

invention relates to the use of fragments of noggin, chordin, cerberus 1 homolog, gremlin, and DAN as BMP-2 inhibitors.

[0019]   Another embodiment of this invention provides a method for treating cancer by administering to a patient a therapeutically effective amount of an expression vector encoding a BMP-2 inhibitor, such as a polypeptide that binds BMP-2 or an antisense oligonucleotide that binds to the nucleic acid for BMP-2. Another aspect of this invention includes the expression vector described above in which the nucleic acid sequence for BMP-2 is operably linked to a selective promoter. One preferred selective promoter encompassed by this invention is carcinoembryonic antigen promoter.

[0020]   This invention also encompasses a kit that includes packaging material, a BMP-2 activity inhibitor, and instructions that indicate that the compound can be used for treating cancer in a patient. One type of cancer that may be treated is carcinoma. Particular carcinomas encompassed by this invention are lung cancer, bladder cancer, breast cancer, colon cancer, kidney cancer, ovarian cancer, thyroid cancer, endometrial cancer, omental cancer, testicular cancer, and liver cancer.

[0021]   The diagnostic component of this invention includes a method for diagnosing cancer in a patient by obtaining a biological sample from a patient and measuring the level of BMP-2 in the biological sample, with an elevated level of BMP-2 indicating an increased likelihood of cancer in the patient.

[0022]   Any assay available to measure BMP-2 levels is encompassed by this invention. Particularly preferred are immunoassays. Some examples of immunoassays included in this invention are Enzyme-Linked Immunosorbent Assay (ELISA), Western

blot, immunoprecipitation, in situ immunohistochemistry, and immunofluorescence. ELISA is most particularly preferred.

[0023]   Another aspect of this invention is a method for the diagnosis of cancer in a patient by detecting overexpression of BMP-2 in the patient by (i) quantifying in vivo or in vitro the presence of BMP-2 in a patient or a biological sample obtained from a patient, (ii) comparing the result obtained in step (i) to that of a normal, non-cancerous patient, and (iii) diagnosing the presence of cancer based on an increased level of BMP-2 in step (ii) relative to a normal, non-cancerous patient.

[0024]   An additional aspect of the present invention demonstrates that BMP-2 stimulates lung tumorigenesis by stimulating angiogenesis of developing tumors. Furthermore, BMP-2 induced angiogenesis involves a direct activation of human endothelial cells. The present invention has discovered that vascular endothelial growth factor (VEGF), which is an active target in cancer therapy, stimulates BMP-2. Thus, BMP-2 enhances tumorigenesis by mediating an angiogenic response.

[0025]   A therapeutic aspect of the invention comprises administering a therapeutically effective amount of a BMP-2 activity inhibitor to a patient to treat tumors or to treat the risk of developing tumors in a patient by decreasing vascular development and/or angiogenesis. Preferably, the BMP-2 activity inhibitor is administered in a therapeutically effective carrier and the administration is continued until the tumor or risk of the tumor is treated.

[0026]   BMP-2 also mediates robust tube formation in both human aortic endothelial cells and human umbilical vein endothelial cells, which demonstrates its role in inducing

endothelial differentiation. BMP-2 activates cancer cells to express Id, cyclin E,

phosphorylation of Rb and Erk 1/2. BMP-2 also activates PI-3, MEK, and p38, all of

which are involved in pathways involved in the transformation of cancers. Thus, another

aspect of the present invention comprises a method of decreasing expression of Id,

cyclin E, and phosphorylation of Rb and Erk 1/2 in cancer cells or precancerous cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027]   Figure 1 illustrates representational difference analysis (RDA) subtraction.

Figure 1(a) shows amplification of cDNA prior to subtraction. Lane 1: IHBE cells; lane

2: lung carcinoma. Figure 1(b) shows the distinct cDNA bands present after the second

round of subtraction and amplification. Figure 1(c) lists the proteins that were identified

by a BLAST data base search after the DNA corresponding to each of the bands shown in

Figure 1(b) was isolated and sequenced.

[0028]   Figure 2 is an ethidium-stained agarose gel showing the results of RT-PCR

performed on human lung cancer specimens. Lanes 1-4 contain the results of the RT-

PCR of various specimens, while lane 5 contains a marker.

[0029]   Figure 3 illustrates Western blots showing mature BMP-2 overexpressed in

lung cancer tissue specimens and lung cancer cell lines. Figure 3(a) is a representative

Western blot showing overexpression of BMP-2 in cancer tissue specimens. Lanes 1-5:

normal lung tissue, lane 6: SOAS osteosarcoma cell line, lanes 7-11: non-small lung cell

carcinomas. Figure 3(b) is the corresponding actin immunoblot. Figure 3(c) is a Western

blot of non-small cell lung carcinoma (NSCLC) subtypes. Lanes 1-4: normal lung tissue,

lane 5: squamous carcinoma, lane 6: adenocarcinoma, lane 7: bronchoalveolar carcinoma, lane 8: large cell carcinoma. Figure 3(d) is the actin immunoblot corresponding to Figure 3(c). Figure 3(e) is a BMP-2 immunoblot of lane 1: benign lung tumor, lane 2: mesthotheleoma, lane 3: normal lung tissue, lane 4: carcinoid tumor, lane 5: normal lung, lane 6: NSCLC, lane 7: normal lung tissue, lane 8: NSCLC, lane 9: recombinant BMP-4. Figure 3(f) is a BMP-4-probed Western blot with the same lane contents as Figure 3(e), except lane 9, which is recombinant BMP-4. Figure 3(g) is the actin immunoblot corresponding to Figure 3(f).

[0030] Figure 4(a) is a Western immunoblot of total cellular protein that demonstrates that normal and malignant human lung cell lines express mature BMP-2 protein. Lanes (1) IHBE; (2) SOAS; (3) H7249; (4) A549. Figure 4(b) is a Western blot of cell culture media showing lung cancer cell lines secrete a BMP-2 precursor protein. The lanes are: (1) lung cancer tumor specimen; (2) A549 media; (3) H7249 media; (4) IHBE; (5), NBE media; (6) serum free media alone. Figure 4(c) is an immunoblot of BMP type IA receptor. Lanes (1-3) normal lung tissue specimens; (4) IHBE cells; (5) H7249 cells; (6) A549 cells; (7-9) lung cancer tissue specimens. Figure 4(d) is an immunoblot of BMP type 1B receptor. (1-3) normal lung tissue specimens; (4) IHBE cells; (5) H7249 cells; (6) A549 cells; (7-9) lung cancer tissue specimens.

[0031] Figure 5(a) is an immunohistochemistry localizing BMP-2 expression to the tumor cells. BMP-2 expression in a NSCLC demonstrating cytoplasmic staining of the tumor cells (arrowheads). The nuclei (n) of the tumor cells and the interstitium (I) are non-reactive. Figure 5(b) shows preabsorption of the BMP-2 antibody with recombinant

human BMP-2 is non-reactive with the tumor cells (arrows). Original magnification is 82X.

[0032]   Figure 6(a) is a BMP-2 Western blot of human breast tumors and corresponding normal tissue. Lane 1: NSCLC, lanes 2-5: breast carcinomas, lanes 6-8: normal breast tissue, lane 9: recombinant BMP-2. Figure 6(b) is a BMP-2 Western blot of common human carcinomas and the corresponding normal tissue. Lane 1: normal endometrium, lane 2: endometrial carcinoma, lane 3: ovarian carcinoma, lane 4: normal colon, lane 5: colon carcinoma, lane 6: normal bladder, lane 7: bladder carcinoma.

[0033]   Figure 7(a) is a Western blot showing BMP-2 expression in metastatic tumors. Lane 1: interstitial inflammatory lung disease, lane 2: normal omentum, lane 3: metastatic kidney tumor, lane 4: normal lymph node, lane 5: metastatic breast cancer, lane 6: metastatic kidney tumor, lane 7: metastatic NSCLC, lane 8: omentum carcinoma. Figure 7(b) is the corresponding actin immunoblot. Figure 7(c) is a BMPR IA Western blot, while Figure 7(d) is a BMPR IB Western blot. The contents of the lanes on both blots are the same: lane 1: normal kidney, lanes 2-3: normal lung, lane 4: metastatic kidney carcinoma, lane 5: metastatic breast carcinoma, lane 6: metastatic NSCLC, lanes 7-9: NSCLC. Figure 7(e) is BMPR IA Western blot and Figure 7(f) is a BMPR IB Western blot of common human carcinomas. Lane contents are the same on both blots: lane 1: normal kidney, lane 2: normal endometrium, lane 3: omentum, lane 4: normal colon, lane 5: ovarian carcinoma, lane 6: kidney carcinoma, lane 7: endometrial carcinoma, lane 8: omental tumor, lane 9: colon carcinoma.

[0034]   Figure 8 is a Western blot showing BMP-2 in serum samples from lung cancer patients. Lanes 1-2: serum samples, lane 3: recombinant BMP-2. 14kD is the size of mature BMP-2.

[0035]   Figure 9 shows that secreted BMP-2 precursor is proteolytically cleaved by human leukocytes. Cell culture media from the A549 cells incubated with leukocytes for 16 hours is probed with BMP-2 antibody recognizing its mature C-terminal end. Figure 9(a) is the resulting Western blot: lane 1: A549 lysate, lane 2: media without leukocytes, lanes 3-4: media with human leukocytes. Figure 9(b) is the same immunoblot hybridized with BMP-2 antibody recognizing its N-terminal end. Figure 9(c) is a Western blot of leukocyte samples probed with anti-furin antibody.

[0036]   Figure 10 shows that BMP-2 treatment enhances formation of blood vessels around a cancerous tumor. Each picture is of tissue from a nude mouse injected either with A549 cells or with A549 cells and BMP-2. The picture in the upper right shows a tumor and surrounding tissue from a nude mouse injected with A549 cells. Upper left: control. Upper right: mouse treated with BMP-2. Lower left: mouse treated with noggin.

[0037]   Figure 11 shows tissue from nude mice injected with A549 cells and nude mice co-injected with A549 cells and BMP-2 stained with anti-CD 31 antibody, which recognizes endothelial cells, viewed from under a microscope. Figure 11(a) on the left is the control. Figure 11(b) on the right is BMP-2 treated tissue.

[0038]   Figure 12 shows that BMP-2 regulates sonic hedgehog expression. Figure 12(a) is a Western blot that was probed with anti-sonic hedgehog and shows an increase in sonic hedgehog expression as the amount of recombinant BMP-2 added to the A549 cell culture

is increased. Lane 1 is A549 lysate and 100 ng of BMP. Lane 2 is A549 lysate and 10 ng

of BMP. Lane 3 is A549 lysate and 1 ng of BMP. Lane 4 is A549 lysate and 500 pg of

BMP. Lane 5 is A549 lysate without any BMP. The Western blot on the right, Figure

12(b), was probed with anti- sonic hedgehog and shows A549 cell culture media without

added noggin (Lane 1) and cell culture media with added noggin (Lane 2).

[0039]   Figure 13 shows that BMP-2 stimulates the migration of A549 and H7249

human lung cancer cell lines. Figure 13(a): Recombinant human BMP-2, 1 ng/ml, 10

ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml was added to the lower well of the transwell

chamber. Migrated cells were counted using fluorescent microscopy. Figure 13(b): Noggin

inhibits BMP-2 induced migration. Lane (1), media alone; (2) recombinant BMP-2 (500

ng/ml); (3) noggin (10 mg/ml) and recombinant BMP-2 (500 ng/ml). Figure 13(c): H7249

cells migrated off cover slips towards Affi-Blue agarose beads containing recombinant

BMP-2. Figure 13(d): H7249 cells did not migrate off cover slips toward AffiBlue agarose

beads containing dilution buffer. Similar results were found using the A549 cells. All the

above experiments were repeated at least 3 times. Data is presented as mean ± standard

deviation. Figure 13(e): Recombinant human BMP-2 stimulates the invasion of A549 or

H7249 cells. Recombinant BMP-2, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000

ng/ml was added to the lower wells of a Matrigel invasion chamber. Experiments were

repeated at least 3 times. Data was presented as mean ± 5 standard deviation.

[0040]   Figure 14 shows tumor growth after 19 days following the subcutaneous co-

injection of A549 lung cancer cells into nude mice with Affi-blue agarose beads coated with

(1) 100 ug/ml of albumin, (2) recombinant human BMP-2, or (3) recombinant mouse

noggin.

[0041]   Figure 15 shows that noggin inhibits VEGF expression in the A549 lung cancer

cell line. The Western blot, Figure 15(a), was probed with anti-VEGF antibody. The lane

labeled with a plus was cell culture media from cultures treated with noggin. The lane

labeled with a minus was cell culture media from control cultures. Figure 15(b) is a chart

showing the results of an ELISA.

[0042]   Figure 16 is a photograph showing excised mouse lungs. The upper set of lungs

is controls. The lower set of lungs contains A549 cells with forced expression of BMP-2,

which are filled with tumors.

[0043]   Figure 17 shows that BMP-2 stimulates tumor neovasculature. A549 cells were

co-injected subcutaneously into nude mice with Affi-Blue agarose beads coated with (A)

BSA or (B) recombinant human BMP-2. Bold arrow highlights the tumor and smaller

arrow a blood vessel feeding the tumor. Endothelial cells within the tumors were

measured by immunohistochemistry. Photograph of representative immunohistochemical

study showing blood vessels in tumors treated with (C) BSA or (D) recombinant BMP-2.

Arrow demonstrating blood vessels that are staining brown. (E) Data showing the number

of blood vessels per 40X field in tumors treated for 4 -6 or 12-14 days. Data depicts the

average of at least 3 experiments per time point. * p <0.05

[0044]   Figure 18 is a Matrigel angiogenesis assay. (A) Photograph of Matrigel plugs

containing A549 cells supplemented with (A 1) BSA or (A 2) recombinant BMP-2 or (A

3) Matrigel plug containing A549-AS cells alone (AS). (B) Representative photograph of

an immunohistochemical study demonstrating endothelial cells within the Matrigel plugs. Blood vessels are staining brown. Matrigel plugs containing A549 cells supplemented with (B 1) BSA (B 2) recombinant BMP-2 or (3) A549-AS alone. Experiments were repeated 6 times. (C) Graphic representation of the number of blood vessels per high-powered field. Also shown is the number of blood vessels in Matrigel plugs containing BMP-2 preincubated with recombinant noggin and A549 cells transfected with pcDNA3 (vector). * $p < 0.05$ compared to control.

[0045]   Figure 19 is four blots demonstrating BMP-2 activates endothelial cells. (A) Western blot analysis was performed on cell lysate from HAEC and probed with antibodies specific for BMP receptors IA, IB, and II. (B) HAEC and (C) HUVEC were treated with recombinant BMP-2 and cell lysates were subjected to Western blot analysis using antibodies specific for phosphorylated Smad1, ID1, and phosphorylated ERK-1/2. An anti-actin antibody was used as a loading control. Experiments were repeated at least 3 times. (D) HAEC protein was precipitated with an anti-Smad 1/5/8 antibody and Western blot probed with an anti-phosphoserine antibody. HAEC were treated with (1) recombinant BMP-2 or (2) vehicle control.

[0046]   Figure 20: BMP-2 Induces Nuclear Accumulation of Smad 1/5/8. Localization of Smad 1, 5 and 8 was determined by immunofluorescence microscopy. HAEC or HUVEC were treated for 30 minutes with I(a) vehicle control I(b) recombinant BMP-2 I(c) recombinant BMP-2 and recombinant noggin or I(d) anti-Smad 1/5/8 antibody with blocking peptide. Figure 20(b): II. HAEC or HUVEC were treated with recombinant BMP-2 for the designated time points. (II A) Imaging using differential interference

contrast microscopy and (II B) immunofluorescent microscopy. (II C) The percentage of

HAEC or HUVEC demonstrating nuclear staining after BMP-2 treatment. A minimum of

100 cells were counted. Each study was performed at least 3 times.

[0047]   Figure 21 is a series of three bar graphs illustrating that BMP-2 stimulates

proliferation, but not migration of endothelial cells. (A) Migration of HAEC treated with

vehicle control, BMP-2, or VEGF. The rate of DNA synthesis was determined by

measuring incorporated H3-thymidine. (B) The rate of DNA synthesis of HAEC treated

with recombinant BMP-2. (C) The rate of DNA synthesis of HUVEC treated with

recombinant BMP-2. Values reported represent the average of 3 independent

experiments performed in duplicate. * $p < 0.05$.

[0048]   Figure 22: BMP-2 Stimulates Tube Formation of Endothelial Cells.

Representative photograph of tube-like structures formed by HAEC treated with (A)

vehicle control (B) 500pg/ml BMP-2 (C) 1ng/ml BMP-2 or (D) BMP-2 pre-incubated

with noggin. Original magnification was a 4X. Graphic representation of the number of

tubes per low powered field for (E) HAEC and (F) HUVEC. Each condition was repeated

at least 3 times in duplicate. * $p < 0.05$ compared to control.

[0049]   Figure 23 shows the A549 cell proliferation for both 6 and 24 hours in SFM

and FCS with varying concentrations of BMP administered.

[0050]   Figure 24 is a chart of the percentage of cells with Smad 1/5/8 nuclear staining

in two types of media, 5% FCS and LHC-8 SFM, for 15, 30, and 120 minutes. A vehicle

is also added for control purposes.

[0051]   Figure 25 is a bar graph representing KI-67 staining in mice tumors for a control and BMP-2 as measured in cells/40X field.

[0052]   Figure 26 is a photograph of the cells stained in Figure 25, with both the control cells and BMP-2 cells shown.

DETAILED DESCRIPTION OF THE INVENTION

[0053]   The present invention is related to the present discovery that the overexpression of bone morphogenetic protein-2 (BMP-2) is linked to cancer invasion and growth. BMP-2 is overexpressed in many common human cancers and regulates molecular pathways that are involved in the promotion of cancer. Inhibiting BMP-2 activity reduces the size of cancerous tumors in nude mice and down regulates the expression of VEGF and sonic hedgehog, both known cancer promoters, in lung cancer cell lines. Thus, the present invention is directed toward using BMP-2 as a target for cancer treatment therapies and as a means to diagnose cancer.

[0054]   The therapeutic component of this invention involves administering to a patient a composition that inhibits bone morphogenetic protein-2 activity. Such inhibition may be accomplished by ligands or antibodies that bind to BMP-2 or BMP-2 receptors. It may also be achieved by preventing the processing of pro-BMP-2, or blocking transcription or replication of BMP-2 DNA or translation of BMP-2 mRNA. Delivery of such compositions may be systemic or tissue-targeted. Additionally, decreased vascularization and decreased tumor expression may be achieved by decreasing the expression of Id,

cyclin E, and phosphorylation of Rb and Erk 1/2 and controlling the activity of other tumor enhancing genes.

[0055] The diagnostic component of the invention involves measuring the BMP-2 level in biological samples from a patient and determining whether that level is elevated from a normal level. A related aspect of the invention involves measuring the BMP-2 level in biological samples from both a patient and a non-cancerous subject and comparing those levels. Presumably, the levels of BMP-2 in the non-cancerous subject are physiologically normal levels. Preferably, the levels are taken from a study of non-cancerous subjects of a similar age and the level of BMP-2 is from a similar or the same organ, fluid or bodily location. Elevated levels of BMP-2 in the patient compared to the non-cancerous subject indicate cancer.

[0056] Although specific embodiments of the present invention will now be described, it should be understood that such embodiments are examples that are merely illustrative of a small number of the many possible specific embodiments that can represent applications of the principles of the present invention. Various modifications obvious to one skilled in the art to which the present invention pertains are within the spirit, scope and contemplation of the present invention as further defined in the appended claims.

DEFINITIONS

[0057] A "bone morphogenetic protein-2 activity inhibitor" is a composition that antagonizes the activity of the BMP-2 protein by specifically binding to it or to BMP

receptors, blocks the activation of pro-BMP-2, prevents the replication or transcription of the BMP-2 gene, or the translation of BMP-2 mRNA into protein.

[0058]   "Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. Polypeptide refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques that are well known in the art. Such modifications are well-described in basic texts and in more detailed monographs, as well as in a voluminous research literature.

[0059]   An "expression vector" is a recombinant vector that incorporates the desired gene and associated control sequences that promote and/or regulate expression of the gene. The desired gene is "operably linked" to such control sequences. The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in an appropriate position in the expression vector relative to the coding sequence so as to enable expression of the coding sequence. The preparation of such recombinant expression vectors as well as the use of various control sequences is well known to those of skill in the art and described in many references. See, for example, Sambrook, J., et al., Molecular Cloning: A Laboratory Manual 2nd ed. (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory) (1989).

[0060]   A "selective promoter" refers to a promoter that is not indiscriminately expressed. Instead, it may only be expressed in certain tissues, certain tumors, in

response to certain treatments, or in response to certain events in a cell. Such tissue specific, tumor-selective, treatment-responsive, or tumor endothelium directed promoters are described in Nettlebeck, D.M., et al., "Gene therapy: designer promoters for tumour targeting" Trends Genet 16(4); 174-81 (2000).

[0061]   An "expression vector vehicle" refers to an expression vector paired with a moiety that facilitates delivery of the expression construct to cells *in vivo*. An expression vector may incorporate genes encoding the delivery moiety. One example of such an expression vector is a viral vector.

[0062]   The term "antibody" refers to polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of a Fab or other immunoglobulin expression library.

[0063]   "Polyclonal" refers to antibodies that are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen or antigenic functional derivative thereof. For the production of polyclonal antibodies, various host animals may be immunized by injection with the antigen. Various adjuvants may be used to increase the immunological response, especially when using an entire protein, or a larger section of the protein. The type of adjuvant used will depend on the hosts. Typical adjuvants include Fruend's, Fruend's complete, or oil-in-water emulsions. In these cases, the entire protein or portion thereof can serve as the antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the antigen. Commonly

utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH).

[0064]   "Monoclonal antibodies" are substantially homogeneous populations of antibodies to a particular antigen. They may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. Such methods are well known to those of ordinary skill in the art and include general hybridoma methods of Kohler and Milstein, Nature (1975) 256: 495-497, the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4:72 (1983) and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, pp.77-96, Alan R. Liss, Inc. (1985). The basic technique involves injecting a mouse, or other suitable animal, with an antigen. The animal is subsequently sacrificed and cells taken from its spleen are fused with myeloma cells. The result is a hybrid cell, referred to as a hybridoma, which reproduces in vitro. The population of hybridomas is screened to isolate individual clones, each of which secretes a single antibody species to the antigen. The individual antibody species obtained in this way are each the product of a single B cell from the immune animal generated in response to a specific antigenic site recognized on the antigen. Kohler, G. and Milstein, C. Nature (London) 256: 495-497 (1975) and Eur. J. Immunol. 6: 511-519 (1976).

[0065]   The term "antibody fragment" refers to a portion of an antibody, often the hyper variable region and portions of the surrounding heavy and light chains, that displays specific binding affinity for a particular molecule. The term antibody fragment also includes single chain antibodies.

[0066]   An "antisense oligonucleotide" is an oligonucleotide that specifically

hybridizes, under cellular conditions, with the cellular mRNA or genomic DNA encoding

a BMP-2 protein or some portion of such cellular or genomic DNA, thereby inhibiting

biosynthesis of the BMP-2 protein. The binding may be via conventional base pair

complementarity, or, in the case of binding to DNA duplexes, via specific interactions in

the major groove of the double helix.

[0067]   The term "effective amount" refers to the quantity of a compound that is

sufficient to yield a desired therapeutic response without undue adverse side effects (such

as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk

ratio when used in the manner of this invention. The specific "effective amount" will

obviously vary with such factors as the particular cancer being treated, the physical

condition of the patient, the type of mammal being treated, the duration of the treatment,

the nature of any concurrent therapy, the specific formulations employed, and the

structure of the compounds or their derivatives.

[0068]   A "patient" is a mammal suspected of having cancer. The patient is preferably

human but may also be another mammal, such as a cat, dog, horse, cow, rat, or mouse.

[0069]   A "biological sample" is a substance obtained from the patient's body. The

particular "biological sample" selected will vary based on the purpose and/or assay for

which the sample is obtained. For example, if a patient is suspected or known to have

cancer, the sample will be the substance most likely to contain cancer. If the sample is

taken from a person without cancer, the sample will likely be the same substance that is

taken from a patient as a basis for comparison. An "elevated level" means the level of

bone morphogenetic protein-2 that is greater than the level of analyte present in a

particular biological sample of patient that is not suffering from cancer.

[0070]   A "carcinoma" is an epithelial cancer. Examples of carcinomas are bladder

cancer, breast cancer, colon cancer, kidney cancer, lung cancer, ovarian cancer, thyroid

cancer, endometrial cancer, omental cancer, testicular cancer, and liver cancer. The

epithelium predominately lines ducts and lining of organs or glands.

BMP-2 AS A TARGET IN THE TREATMENT OF CANCER

[0071]   The present invention is directed to the use of BMP-2 as a target in the

treatment of cancer. Amino acids #283-396 of SEQ ID NO: 2 constitute the amino acid

sequence of mature human BMP-2. Nucleotides #372-1514 of SEQ ID NO: 1 constitute

the nucleotide coding sequence for human BMP-2. Any composition that specifically

binds BMP-2 or a BMP-2 receptor, thereby antagonizing BMP-2 activity, blocks the

processing of pro-BMP-2, and/or prevents the replication or transcription of BMP-2 DNA

or the translation of BMP-2 mRNA could be used as a therapy to treat cancer.

[0072]   A compound that specifically binds to BMP-2 is any compound such as a

polypeptide or an antibody that has a binding affinity for any naturally occurring isoform,

splice variant, or polymorphism of BMP-2. As one of ordinary skill in the art will

appreciate, such "specific" binding compounds may also bind to other closely related

proteins that exhibit significant homology (such as greater than 90% identity, more

preferably greater than 95% identity, and most preferably greater than 99% identity) with

the amino acid sequence of BMP-2. Any compound that binds to BMP-2 sufficiently to suppress BMP-2 activity is contemplated by the present invention.

[0073]   Similarly, a compound that specifically binds to a BMP receptor is any compound that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of the BMP receptor. As one of ordinary skill in the art will appreciate, such "specific" binding compounds may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of a BMP receptor. The present invention embodies polypeptides that specifically bind to BMP-2, thereby inhibiting its activity, or that specifically bind to BMP receptors, thereby inhibiting BMP-2 activity. Specific embodiments of such polypeptides are described below.

[0074]   The present invention encompasses known antagonists of BMP-2 activity, including noggin (35) (US Patent No. 6,075,007, Economides, et al.), chordin (36) (US Patent No. 5,896,056), gremlin (GenBank Accession No. AF 154054), cerberus 1 homolog (GenBank Accession No. NM-005454), and DAN.

[0075]   Recombinant mouse noggin from R & D Systems (Minneapolis, MN) was used in the inhibition experiments described in the Results section below. Mouse and human noggin share 98% homology. Therefore, this invention also relates to use of a polypeptide with the amino acid sequence of mature mouse noggin (amino acids #20-231 of SEQ ID NO: 6) and with the amino acid sequence of mature human noggin (amino acids #20-231 of SEQ ID NO: 4) as a BMP-2 activity inhibitor. The amino acid sequence

for human chordin is SEQ ID NO: 8, for human gremlin is SEQ ID NO: 10, and for

cerberus 1 homolog is SEQ ID NO: 12. The nucleotide coding sequence for human

noggin is SEQ ID NO: 3, for mouse noggin is SEQ ID NO: 5, for human chordin is

nucleotides #247-3114 of SEQ ID NO: 7, for human gremlin is nucleotides #130-684 of

SEQ ID NO: 9, for human cerberus 1 homolog is SEQ ID NO: 11.

[0076]   This invention also embodies polypeptide fragments of noggin, chordin,

gremlin, cerberus1 homolog, and DAN that bind BMP-2 and inhibit its activity. Such

polypeptides may be tested for inhibitory efficiency by culturing cells transformed with

progressively shorter portions of the nucleotide sequences encoding the above proteins,

recovering and purifying from the various cultures the resulting polypeptide, and testing

those polypeptides for their ability to inhibit BMP-2 activity.

[0077]   This invention also includes genetically altered BMP receptor proteins that

inhibit BMP-2 activity. For example, altered BMP receptors that inhibit the binding

effects of BMP-2 are described in U.S. Patent No. 6,291,206 (Wozney, et al.).

[0078]   Also included by this invention are polypeptides that bind BMP receptors

without activating them. (37, 38) Particularly preferred are ligands that will bind BMP IB

receptors, which are a subtype of BMP receptors. Aberrant expression of the BMP IB

receptor in many human cancer specimens has been noted, as discussed in the Results

section below. (39) The coding sequence for BMP IB precursor is nucleotides #274-1782

of SEQ ID NO: 13. The amino acid sequence for BMP IB is amino acids #14-502 of

SEQ ID NO: 14.

[0079]   This invention also encompasses expression vectors that incorporate a

nucleotide sequence encoding an inhibitor of BMP-2 activity operably linked to control

sequences that promote and/or regulate expression of the nucleotide sequence. The

preparation of such expression vectors, as well as the use of various control sequences, is

well known to those of skill in the art and is described in many references, such as

Sambrook, et al. (1989). Expression vectors can be derived from bacterial plasmids, from

bacteriophages, from transposons, from yeast episomes, from insertion elements, from

yeast chromosomal elements, from viruses and from combinations thereof, such as those

derived from plasmid and bacteriophage genetic elements, such as cosmids and

phagemids. Promoters can be prokaryotic, such as lacI, lacZ, T3, T7, gpt, lambda PR,

PL, and trp, or eukaryotic, such as CMV immediate early, HSV thymidine kinase, early

and late SV40, LTR's from retrovirus, and mouse metallothionein-1. Selective promoters

such as those described in Nettlebeck, D.M., et al., "Gene therapy: designer promoters for

tumour targeting" Trends Genet 16(4); 174-81 (2000) that are tissue-specific, tumor-

selective, treatment-responsive, or tumor endothelium directed may also be used. For

example, the promoter of the carcinoembryonic antigen (CEA) is expressed on many

breast, lung, and colorectal cancers.

[0080]   For introduction of a gene that encodes a protein that antagonizes BMP-2

activity, an expression vector vehicle that will facilitate delivery of the desired gene to the

affected cells may be used. One way to facilitate delivery is by using an expression

vector derived from virus. Examples of viral vectors that have been successfully used to

deliver desired sequences to cells with high infection efficiency are adenoviral, retroviral,

vaccinia viral, and adeno-associated viral vectors. Commonly used viral promoters for expression vectors are derived from polyoma, cytomegalovirus, Adenovirus, and Simian Virus 40 (SV40). It is also possible to use promoter or control sequences normally associated with the desired gene sequence, if such control sequences are compatible with the host cell systems.

[0081]   Non-viral expression vector vehicles are also available. For instance, the expression vector could be associated with one or more lipids. As is known in the art of lipid-based gene delivery, such nucleic acid-lipid complexes can be in a variety of different forms depending generally on the nature of the lipid employed, the ratio of nucleic acid to lipid and/or other possible components, and the method by which the complex is formed. Examples of complexes include liposomes and micelles. Liposome-mediated gene transfer seems to have great potential for certain in vivo applications in animals. Studies have shown that intravenously injected liposomes are taken up essentially in the liver and the spleen, by the macrophages of the reticuloendothelial system. Using a catheter to introduce liposomes coupled to expression vectors to particular cellular sites has also been described. (Nabel, E.G., et al., Science 249:1285-1288 (1990))

[0082]   Another possible expression vector vehicle consists of a cell receptor-specific ligand and a DNA-binding agent that would bind to the expression vector. (Nishikawa, M. et al., Gene Therapy 7:548-55 (2000)). Such a vehicle could also comprise a cell receptor-specific ligand and the nucleic acid-lipid complex described above. (Nicolau, C. et al., Methods Enzymol 149: 157-76 (1987))

[0083]   In addition, the present invention embodies antibodies that specifically bind

BMP-2 or BMP receptors, thereby inhibiting BMP-2 activity. When raising antibodies to

BMP-2 or BMP receptors, the entire protein (either the precursor or the processed

protein), or a portion thereof, may be utilized. Information useful in designing an antigen

for the production of antibodies to BMP-2 may be deduced by those of skill in the art by

homology analysis of SEQ ID NO: 2, especially amino acids #283-396 of SEQ ID NO: 2.

[0084]   A recombinant human BMP-2 protein is commercially available from R & D

Systems (Minneapolis, MN) and portions of the BMP-2 protein may be produced by a

variety of methods. In order to raise antibodies to particular epitopes, peptides derived

from the full BMP-2 sequence may be used. Custom-synthesized peptides in the range of

10-20 amino acids are available from a multitude of vendors, and can be ordered

conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be

synthesized by solid-phase methods, or may be recombinantly produced in a recombinant

protein production system. In order to ensure proper protein glycosylation and processing

an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is

preferred.

[0085]   Selection of antibodies which alter the activity of BMP-2 may be accomplished

in several ways. Antibodies that alter the binding of BMP-2 to a receptor may be

detected by well-known binding inhibition assays. For instance, according to standard

techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to

BMP-2, which has been immobilized in a microtiter well, is assayed for BMP-2 binding

in both the presence and absence of the appropriate receptor. The decrease in binding

will be indicative of a competitive inhibitor relationship between the antibody and the receptor. In addition, antibodies that are useful for altering the function of BMP-2 may be assayed in functional formats, such as the cell migration assays described in the Results and Examples sections.

[0086]   This invention also embodies compositions that prevent the processing of inactive BMP-2 precursors. BMP precursors are proteolytically activated by proprotein convertases. For example, BMP-2 is cleaved by furin convertase from human leukocytes Furin inhibitors are known. (40) While the BMP-2 inhibitors discussed above adversely affect BMP-2 activity after it is expressed, it will be readily apparent to one of ordinary skill in the art that specific prevention of BMP-2 biosynthesis will achieve the same goals as more direct inhibition of its activity. Consequently, this invention also encompasses inhibition of BMP-2 biosynthesis as a method for treating cancer. Such inhibition may be achieved by selectively degrading mRNA encoding BMP-2 or BMP-4 or by interfering with transcription or translation of such mRNA. (41) As mentioned above, BMP-2 shares 92% homology with BMP-4.

[0087]   Inhibition of BMP-2 biosynthesis to treat for cancer could also be achieved through antisense therapy. Antisense therapy is the administration or in situ generation of oligonucleotides that specifically hybridize, under cellular conditions, with the cellular mRNA or genomic DNA encoding a BMP-2 protein or some portion of such cellular or genomic DNA, thereby inhibiting biosynthesis of the BMP-2 protein. Antisense therapy refers generally to the range of techniques known by one of ordinary skill in the art, and includes any therapy that relies on specific binding to oligonucleotide sequences.

[0088]   Delivery of an antisense oligonucleotide of the present invention can occur in a

variety of ways. For example, an antisense oligonucleotide can be delivered as an

expression vector that produces RNA, which is complementary to at least a unique

portion of the cellular mRNA encoding BMP-2. Such an expression vector could be

delivered to cells by one of the expression vector vehicles described above.

Alternatively, the antisense oligonucleotide could be generated ex vivo as an

oligonucleotide probe which, when introduced to the cell, inhibits biosynthesis of BMP-2

proteins by hybridizing with the mRNA or genomic sequences encoding BMP-2. Such

oligonucleotide probes could be modified oligonucleotides that are resistant to

endogenous nucleases and therefore are stable in vivo. General methods to construct

oligomers useful in antisense therapy are known in the art. (Van der krol, et al.,

Biotechniques 6:958-976 (1988); Stein, et al., Cancer Res. 48:2659-2668 (1988).

[0089]   Dosage forms of the BMP-2 inhibitors of this invention include

pharmaceutically acceptable carriers known to those of ordinary skill in the art.

Pharmaceutically acceptable components are those that are suitable for use with mammals

without undue adverse side effects (such as toxicity, irritation, and allergic response)

commensurate with a reasonable benefit/risk ratio. The carrier can be a solid or liquid.

The type of carrier is generally chosen based on the type of administration being used.

The active agent can be co-administered in the form of a tablet or capsule, as an

agglomerated powder or in a liquid form. Examples of suitable solid carriers include

lactose, sucrose, gelatin and agar. Capsule or tablets can be easily formulated and can be

made easy to swallow or chew; other solid forms include granules and bulk powders.

Tablets may contain suitable binders, lubricants, diluents, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents and melting agents. Examples of suitable liquid dosage forms include solutions or suspensions in water, pharmaceutically acceptable fats and oils, alcohols or other organic solvents, including esters, emulsions, syrups or elixirs, suspensions, solutions and/or suspension reconstituted from non-effervescent preparations reconstituted from effervescent granules. Such liquid dosage forms may contain, for example, suitable solvents, preservatives, emulsifying agents, suspending agents, diluents, sweeteners, thickeners and melting agents. Parenteral and intravenous forms may also include isotonic salts and other materials to make them compatible with the type of injection or delivery system chosen.

[0090]   For administration of an antibody to BMP-2, the pharmaceutically acceptable carrier will usually be an aqueous solution, such as normal saline or phosphate-buffered saline (PBS), Ringer's solution, lactate-Ringer's solution, or any isotonic physiologically acceptable solution for administration by the chosen means. In addition to additives for adjusting pH or tonicity, the antibody may be stabilized against aggregation and polymerization with amino acids and non-ionic detergents, polysorbate, and polyethylene glycol. Optionally, additional stabilizers may include various physiologically acceptable carbohydrates and salts. In addition, polyvinylpyrrolidone may be added in addition to the amino acid. Suitable therapeutic immunoglobulin solutions, which are stabilized for storage and administration to humans are described in U.S. Patent No. 5,945,098. Other agents, such as human serum albumin (HSA), may be added to the pharmaceutical composition to stabilize the antibody conjugates.

[0091]   The method of administration can be any suitable method that effectively

alleviates the particular tumor and/or cancer being treated. Possible methods of

administration are oral, rectal, parenteral, enteric, subcutaneous, transdermal, peritoneal,

intratumoral, or intravenous.

[0092]   Any suitable dosage of the compounds may be given in the method of the

invention. Dosage levels and requirements are well recognized by those of ordinary skill

in the art. As one of ordinary skill in the art will appreciate, an amount constituting an

effective amount will vary depending on particular factors. For instance, specific dosage

and treatment regimens will depend on facts such as the patient's general health profile,

the type of cancer being treated, the severity and course of the patient's disorder, other

therapeutics being administered to treat the cancer, and the judgment of the treating

physician.

[0093]   The present invention also provides kits for treating cancer using BMP-2

activity inhibitors. For example, such kits can comprise any one or more of the following

materials: packaging material, at least one type of BMP-2 activity inhibitor, and

instructions regarding dosage, method of administration, or the like for using the inhibitor

to treat cancer.

DETECTION OF BMP-2 TO AID IN DIAGNOSIS OF CANCER

[0094]   In addition to its therapeutic aspects, the present invention also relates to a

diagnostic method for detecting the presence of elevated levels of BMP-2 in the patient.

The inventor has shown that BMP-2 is expressed in many common cancers. Elevated

levels of BMP-2 can be detected in various biological samples in mammals, preferably humans. The inventor has further shown the presence of BMP-2 in the blood serum of a human patient with cancer. Biological samples, including but not limited to blood, vitreous humor, sputum, aqueous humor, synovial fluid, urine, ascites, and tissue, will be drawn from the patient using standard techniques. Particularly preferred are serum samples.

[0095] The measurement of BMP-2 levels may be monitored using any method possible to detect BMP-2 in biological samples. Immunoassays, such as Enzyme Linked Immunological Assay (ELISA), Western blots, immunoprecipitation, in situ immunohistochemistry, and immunofluorescence assays are preferred. ELISA is particularly preferred. For a review of general immunoassays, see Stites, D.P., et al., eds., Basic and Clinical Immunology, 8th ed. (Appleton & Lange, Norwalk, Conn.) (1994). Immunological binding assays (or immunoassays) typically use an antibody that specifically binds to a protein of choice, BMP-2, in this case. The antibody is generally fixed to a substrate such as a plate or a column via covalent or non-covalent linkages (e.g., streptavidin, protein A, protein G, secondary antibodies). Immunoassays also often use a labeling agent to specifically bind to and label the complex formed by the antibody and antigen. The labeling agent may be a labeled anti-BMP-2 antibody. Alternatively, the labeling agent may be a third moiety, such as a secondary antibody, that specifically binds to the antibody/antigen complex.

[0096] The immunoassays of this invention may be competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of antigen is directly

measured. In a "sandwich" assay, for example, the anti-BMP-2 antibodies can be bound directly to a solid substrate on which they are immobilized. These immobilized antibodies then capture BMP-2 in the test sample. BMP-2 thus immobilized is then bound by a labeling agent, such as a second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second or third antibody is typically modified with a detectable moiety, such as biotin, to which another molecule specifically binds, e.g., streptavidin, to provide a detectable moiety. Methods of binding molecules to a solid support, either covalently or non-covalently, are well known to those of skill in the art. A variety of solid supports known to those of skill in the art, e.g., plate, columns, dipsticks, membranes, and the like, can be used with the present invention.

[0097]   In competitive assays, the amount of BMP-2 is measured indirectly by measuring the amount of a known modified BMP-2 displaced from a BMP-2 antibody by the unknown BMP-2 in a sample. In one competitive assay, a known amount of modified BMP-2 is added to a sample and the sample is then contacted with an anti-BMP-2 antibody. The amount of known modified BMP-2 bound to the antibody is inversely proportional to the concentration of BMP-2 in the sample. The amount of modified BMP-2 may be detected by providing a labeled modified BMP-2 molecule.

[0098]   The label used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property.

Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, or chemical means. Examples of such labels are magnetic beads, fluorescent dyes, radiolabels, enzymes, and calorimetric labels such as colloidal gold or colored glass or plastic beads.

[0099]   The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule, such as biotin, is covalently bound to the molecule. The ligand then binds to another molecule, such as streptavidin, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize BMP-2. The molecules can also be conjugated directly to a signal-generating compound, e.g., by conjugation with an enzyme or fluorophore.

[00100]   Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge-coupled

devices (CCDs) or photomultipliers or the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally, simple colorimetric labels may be detected simply by observing the color associated with the label.

[00101]   Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies.

RESULTS

[00102]   Experimental results supporting the above uses of BMP-2 and its inhibitors are set forth in detail below. All of the experimental methods mentioned in this section, such as representational difference analysis, Western blot assays, and immunohistochemical studies, are described in detail in the Examples section that follows.

Identification of BMP-2 Using RDA Subtraction Technique

[00103]   Initially, the inventor performed representational difference analysis (RDA) on cDNA derived from normal and cancerous lung tissue samples to identify genes that were uniquely or highly expressed in human lung cancer in comparison to normal tissue. [Fig. 1(a)] RDA has been described in the literature and allows detection of differences in gene expression between two similar populations. It involves exposing digested tester cDNA ligated to a primer to high concentrations of similarly digested but non-primer bearing driver cDNA, melting the tester and driver cDNA, and allowing them to hybridize.

[00104]   Subsequent PCR results in exponential amplification of the target cDNA of the

tester that hybridizes to other tester cDNA. (Hubank, M., Nucleic Acids Research

22:5640-5648 (1994)) Here, a non-small cell lung carcinoma (NSCLC) as the tester and

immortalized human bronchial epithelial (IHBE) cells were used as the driver. IHBE

cells rather than normal lung tissue were used, as IHBE cells proliferate at a rate that is

more similar to human lung carcinomas than to normal lung tissue. Thus, this technique

avoided identifying genes involved in the proliferation cascade but that were not by

themselves transforming.

[00105]   After two rounds of subtraction, several distinct bands, which were cloned and

sequenced, were present in the amplified tester cDNA. [Figure 1(b)] A BLAST database

search identified BMP-2 expression in the lung tumor tissue specimen as well as

expression of alpha-1-antitrypsin, cytokeratin 6, and lambda light. [(Figure 1(c)]

**Expression of BMP-2 In Various Cancer Tissue Specimens, Cancer Cell
   Lines, and Blood Serum from a Cancer Patient**

[00106]   Using reverse transcriptase polymerase chain reaction (RT-PCR), Western

blots, and immunohistochemical assays to study the expression of BMP-2 and its

receptors in various tissue specimens and in cell lines, it was discovered that BMP-2 was

highly expressed in many types of cancers.

[00107]   The initial experiments were performed on normal and cancerous lung tissue

and lung cancer cell lines. RT-PCR was performed using BMP-2 primers and showed

expression of BMP-2 in 9 out of 10 tumors examined. [Figure 2] Using Western blot

analysis, the inventor found that the mature active 14 kD BMP-2 protein was aberrantly

expressed in almost all of the 25 non-small cell lung carcinoma (NSCLC) tissue specimens examined. There was little to no expression of BMP-2 in 11 normal lung tissue specimens. A representative Western blot from that experiment is shown in Figure 3. An anti-actin immunoblot showed near equal loading of the samples, which further confirms the validity of the experiment. [Figure 3(b)] In addition, BMP-2 was found to be highly expressed in all epithelial derived lung carcinomas of which NSCLC is derived and in the rare malignant neuroendocrine tumor. [Figure 3(c) and Figure 3(e), Lane 4, respectively]

[00108]   Western blot analysis of each of the different cell types comprising NSCLC – adeno, squamous, large cell, and bronchoalveolar carcinomas – revealed that the level of BMP-2 expression was not dependent on the cell type or whether the tumor was well or poorly differentiated. In comparison, the level of BMP-2 expression in benign lung tumors [Figure 3(e), Lane 1] and inflammatory diseases of the lung [Figure 3(a), Lane 6] was very low, similar to that seen in normal lung tissue, showing that BMP-2 is not an acute phase protein and that high levels of BMP-2 expression are indicative of malignant tumors. Neither BMP-4 nor BMP-7 expression was detected in the lung tissue specimens or the A549, H7249, IHB, and NBE cell lines by Western analysis. [Figure 3(f)]

[00109]   Tests were also performed to determine whether there was expression of BMP-2 in various lung cancer and normal cell lines. Although the mature BMP-2 protein was detected in the cell lysate of the A549 and H7249 human lung cancer cell lines, the level of expression was not significantly different from the level of expression in the cell lysate of immortalized normal human bronchial epithelial cells (IHBE). [Figure 4(a)] Because

BMP-2 is a secreted protein, its expression in the cell culture media was also examined. A

Western blot of the cell culture media showed the A549 and H7249 cell lines secreted a 43

kD BMP-2 precursor protein. [Figure 4(b), Lanes 2-3] This BMP-2 precursor was not

detected in the media from either the IHBE or normal bronchial epithelial (NBE) cells.

Figure 4(b), Lanes 4-5]

[00110]   Immunohistochemistry studies of patient derived NSCLC also localized the

expression of BMP-2 to the cancer cells. [Figure 5(a)] Absorbing the anti-BMP-2 antibody

with recombinant human BMP-2 completely inhibited staining of the tumors. [Figure 5(b)]

BMP-2 expression was not detected in normal lung tissue by immunohistochemistry.

Expression of 1A and 1B BMP Receptors

[00111]   Next, the inventor found that normal and cancer lung tissue specimens and cell

lines express both type IA and IB BMP receptors. The lung cancer and normal lung

tissue specimens express a 55 kD and 44 kD type IA BMP-2 receptor. The tumor

specimens expressed predominately the 55 kD receptor, while normal lung tissue

specimens expressed a higher percentage of the 44 kD receptor. The A549, H7249, and

IHBE cells only expressed a 44 kD type IA BMP receptor. [Figure 4(c)] The tissue

specimens and cell lines expressed a 44 kD type IB BMP receptor with normal lung tissue

demonstrating more expression than that of the tumor specimens. [Figure 4(d)]

BMP Overexpression in Many Types of Cancerous Tissue

[00112]   Similar to the findings with lung tissue, it was found that BMP-2 was expressed

in many other common human malignancies but not in their corresponding normal

tissues. Western blot analysis revealed that BMP-2 was overexpressed in breast, bladder, colon, endometrial, omental, and kidney carcinomas with low levels of BMP-2 expression in the corresponding normal tissue. [Figures 6(a) and (b)] BMP-2 was also found to be expressed in ovarian [Figure 6(b), lane 3], mesothelioma [Figure 6(e), lane 2], thyroid, hepatoma, and testicular carcinoma.

[00113] BMP-2 and its receptors were also examined in both primary and metastatic carcinomas that were surgically removed from patients. BMP-2 was found to be highly expressed in kidney tumors that had metastasized to the lung, a metastatic breast cancer to chest wall cavity, and a NSCLC lung tumor that had metastasized to a regional lymph node. [Figure 7(a)] The BMP IA receptor was expressed equally between the primary and metastatic carcinomas and the corresponding normal tissue. [Figure 7] The BMP IB receptor was expressed in all metastatic and primary tumors examined. [Figure 7] The BMP IB receptor, in contrast to the BMP IA receptor, was not expressed in all the corresponding normal tissues. While it was expressed in normal lung tissue with slight expression in normal endometrium, it was not expressed in normal kidney, colon, and omentum. [Figure 7(f)] Interestingly, the IB receptor was expressed in both primary and metastatic renal carcinoma, but not in normal kidney tissue. [Figure 7(f), Lane 6] BMP-2 expression was also found in blood serum samples from lung cancer patients. [Figure 8]

Processing of Inactive BMP-2 Precursors

[00114] Because BMP precursors are proteolytically activated by proprotein convertases, the inventor studied whether BMP-2 could be processed following secretion,

hypothesizing that secreted BMP-2 precursors from tumor cells may be processed by cells present in the tumor stroma. Because leukocytes normally infiltrate the lung and because furin convertase is ubiquitously expressed, the ability of leukocytes to cleave proprotein BMP-2 secreted from A549 cells was examined. First, it was determined that the furin convertase is expressed in human leukocytes isolated from whole blood. [Figure 9(c)] Human leukocytes were incubated with A549 cell culture media containing the BMP-2 precursor protein. A Western blot of the incubated media samples was probed with an anti-human BMP-2 precursor antibody that recognizes its C-terminal end. The 45 kD BMP-2 precursor protein was consistently decreased following incubation with the leukocytes. [Figure 9(a)] By probing immunoblots with an anti-human BMP-2 antibody that recognizes its N-terminal end, the inventor identified a 31 kD BMP-2 product present only in the media samples incubated with leukocytes. [Figure 9(b)] This data shows that BMP-2 precursor proteins are cleaved by human leukocytes.

Effect of BMP-2 on Tumors and Cancer Cell Lines

[00115]   After determining that BMP-2 was highly expressed in most common cancers, the inventor performed experiments to show that BMP-2 causes cancer invasion and metastasis. The experiments were conducted with lung cancer cell lines and with nude mice injected with A549 cells.

[00116]   The experiments with the nude mice showed that BMP-2 treatment enhances blood vessel formation around tumors from nude mice injected with A549 cells. Some of the mice were co-injected with BMP-2. Gross observations of tissue harvested after six

days showed that the addition of recombinant BMP-2 to developing tumors in nude mice

caused increased blood vessel formation. [Figure 10] Tissue was also stained with anti-

CD31 antibody which recognizes endothelial cells. A person blind to how the tumors

were created then observed the tissue through a microscope and counted the number of

vessels that had formed in the tumor. This data showed that BMP-2 caused a statistically

significant increase in the number of blood vessels in the tumor. [Figure 11]

[00117]   Other studies showed that addition of BMP-2 to cancer cell lines increased

expression of vascular endothelial growth factor (VEGF) and the oncogene Sonic

Hedgehog. The addition of recombinant BMP-2 to human aortic endothelial cells in

culture caused an increase in VEGF secretion as determined by ELISA performed on the

cell culture media. The concentration of VEGF in the cell culture media before treatment

with BMP-2 was 11.2 pg/ml. The VEGF concentration after treatment with 0.500 pg/ml

BMP-2 was 233.0 pg/ml and after treatment with 1 ng/ml BMP-2 was 2,969.0 pg/ml. The

addition of increasing amounts of BMP-2 to lung A549 lung cancer cells growing in culture

also caused a dose responsive increase in the expression of the oncogene Sonic Hedgehog.

[Figure 12]

[00118]   In addition, the present experiments showed that BMP-2 stimulates the

migration and invasion of the human lung cancer cell lines A549 and H7249. In one

assay, recombinant BMP-2 caused a dose responsive increase in migration of cells from

transwell migration chambers. [Figure 13(a)] In another, BMP-2 stimulated the

migration of A549 and H7249 cells cultured on glass cover slips toward Affi-blue agarose

beads containing recombinant BMP-2. [Figure 13 (c) and (d)] In addition, using

transwell chambers coated with Matrigel, the inventor also showed that recombinant BMP-2 caused a dose responsive increase in the invasion of both A549 and H7249 cells. [Figure 13(e)]

Effects of Inhibiting BMP-2 Expression

[00119]   After finding that BMP-2 enhances cancer invasion and growth, The inventor conducted experiments to determine whether inhibitors of the activity of BMP-2 could be used to treat cancer. In these studies, recombinant mouse noggin (R & D Systems, Minneapolis, MN) was used as a representative inhibitor.

[00120]   The effects of BMP-2 and noggin on tumor growth *in vivo* were examined by co-injecting the A549 cells subcutaneously into nude mice with Affi-Blue agarose beads coated with either albumin, recombinant human BMP-2, or recombinant human noggin. The animals were then sacrificed and tumors measured at 12 or 19 days. Inhibiting BMP-2 activity with noggin resulted in a statistically significant decrease in tumor growth. Addition of BMP-2 resulted in a statistically significant increase in tumor growth. [Figure 14] The inventor also found that noggin completely inhibited the ability of BMP-2 to enhance the migration of the A549 cells in a transwell chamber. [Figure 13(b)] Noggin also decreased the expression of VEGF and sonic hedgehog when added to A549 cells. [Figures 12 and 15]

Mechanism of BMP-2 Action

[00121]   The next set of experiments revealed that BMP-2 significantly enhanced neovascularization of developing tumors. Recombinant BMP-2 greatly enhanced blood

vessel formation in tumors formed from A549 cells injected subcutaneously into athymic

nude mice. Recombinant BMP-2 also stimulated angiogenesis in Matrigel containing

A549 cells in nude mice. The BMP-2 antagonist noggin abrogated BMP-2 induced

angiogenic response. Furthermore, anti-sense transfection of BMP-2 cDNA resulted in a

decrease in blood vessel formation in the Matrigel assays.

[00122]   The present experiments reveal that BMP-2 mediated angiogenesis involves a

direct activation of endothelial cells. BMP-2 induces tube like differentiation of both

human aortic (HAEC) and umbilical vein endothelial cells. BMP-2 also stimulated

proliferation of HAEC. BMP-2 activation of endothelial cells involves a Smad 1/5/8

mediated up-regulation of the proangiogenic factors Id1 and ERK-1/2.

BMP-2 Stimulates Neoangiogenesis In Tumors

[00123]   BMP-2 stimulates angiogenesis in developing tumors, which involves the

direct activation of endothelial cells. Figure 16 is a photograph of excised mouse lungs.

The top set are controls and the bottom set were subjected to A-549 cells and forced

expression of BMP-2. Clearly, the bottom set of lungs is replete of tumors. These data

all support the present discovery that the highly expressed BMP-2 significantly enhances

lung tumorigenesis by stimulating angiogenesis.

[00124]   To assess the effects of BMP-2 on blood vessel formation in developing

tumors, A549 cells were co-injected with Affi-Blue agarose beads coated with

recombinant BMP-2 subcutaneously into athymic nude mice. On visual inspection,

tumors treated with BMP-2 [Figure 17B] had larger and more numerous blood vessels

than those treated with BSA. [See Figure 17A] Immunohistochemistry studies revealed a

6-fold increase in the number of blood vessels that formed within the tumors treated with

BMP-2 [Figure 17D] as opposed to the control. [Figure 17C] The BMP-2 mediated

angiogenic response occurred rapidly demonstrating an increase in blood vessels by the

sixth day that persisted until at least the eighteenth day post injection. Figure 17E is a bar

graph illustrating the blood vessel growth in A549 tumors at varying points in time.

[00125]   To further assess the effects of BMP-2 on tumor neoangiogenesis, A549 cells

were mixed in Matrigel supplemented with recombinant BMP-2 or BSA and injected

subcutaneously into athymic nude mice. Blood vessel formation induced from A549 cells

engineered to secrete less BMP-2 by anti-sense transfection (A549-AS) was also

examined. Overall, Matrigel plugs containing BMP-2 [Figure 18A-2] had more blood

vessels than that of controls [Figure 18A-1]. A549-AS cells showed no evidence of a

neovasculature after 6 days [Figure 18A-3]. Immunohistochemistry also demonstrated

significantly more blood vessels within the Matrigel plugs treated with recombinant

BMP-2 [Figure 18B-2]. Matrigel plugs containing A549-AS cells [Figure 18B-3] had

fewer blood vessels than A549 cells treated with BSA [Figure 18B-1] or A549 cells

transfected with vector alone (vector). [Figure 18C] Inhibiting the activity of BMP-2

with its antagonist noggin, abrogated the angiogenic response induced by recombinant

BMP-2 [Figure 18C].

BMP-2 Activates Smad 1, Id1, and ERK 1/2

[00126] The next experiment examined whether BMP-2 induced angiogenesis involves the activation of human endothelial cells. Western blot analysis showed the expression of BMP-specific receptors in human aortic endothelial cells (HAEC) [Figure 19A]. To determine whether BMP-2 activated BMP-specific Smads, an antibody that recognized the phosphorylated form of Smad1 was used. BMP-2 induced the phosphorylation of Smad1 within 10 minutes in both the HAEC [Figure 19B] and human umbilical vein endothelial cells (HUVEC) [Figure 19C]. BMP-2 mediated Smad phosphorylation persisted for approximately 40 minutes in both endothelial cell lines. To further assess BMP-2 induced Smad phosphorylation, cell lysate from HAEC treated with and without BMP-2 was immunoprecipitated with an antibody recognizing Smads 1,5, and/or 8 and immunoblots were probed with an antibody recognizing phosphorylated serines [Figure 19D]. This study also demonstrated that BMP-2 induces the phosphorylation of BMP-2 specific Smads in endothelial cells. An isotype control antibody did not immunoprecipitate Smads 1,5, and/or 8.

[00127] It is known that BMP-6 activates Id1 in BAEC and MEEK cells. Id is a negative regulator of basic helix loop transcription factors and was shown to regulate endothelial migration and possibly tube formation. The present experiment found that BMP-2 induces a transient increase in the expression of Id1 in both the HAEC [Figure 19B] and HUVEC [Figure 19C].

[00128] Because proangiogenic proteins vascular endothelial growth factor (VEGF), fibroblastic growth factor (FGF), epidermal growth factor (EGF), and angiogenin mediate

a cellular response in endothelial cells through the activation of extracellular signal-regulated kinase (ERK-1/2), this experiment examined whether BMP-2 also activates ERK-1/2. An antibody specific for phosphorylated ERK-1/2 was used in Western Blot analysis. BMP-2 induced a rapid increase in the phosphorylation of ERK-1/2 in both the HAEC and HUVEC. A robust increase was seen in the HAEC that persisted for at least 2 hours [Figure 19B]. Phosphorylation of ERK-1/2 in the HUVEC was not as robust and persisted for only 60 minutes [Figure 19C].

BMP-2 Induced Nuclear Translocation of Smad 1, 5, and/or 8

[00129]   Phosphorylation of Smads 1, 5, and/or 8 stimulates its translocation from the cytoplasm into the nucleus, which then induces transcription of target genes. Using immunofluorescence, the next experiment examined whether BMP-2 stimulates the translocation of Smads 1/5/8 into the nucleus in human endothelial cells. The findings indicate that BMP-2 increases nuclear expression of Smads 1,5, and/or 8 in both HAEC and HUVEC (Figure 20A). Noggin completely antagonized recombinant BMP-2 and induced nuclear accumulation of Smad 1/5/8 [Figure 20A]. Pre-incubating the anti-Smad 1, 5, and/or 8 antibody with a peptide recognized by this antibody abrogated all fluorescent signals [Figure 20A]. Nuclear staining of Smad 1/5/8 corresponded to the BMP-2 induced phosphorylation of Smad 1 found by Western blot analysis. BMP-2 mediated influx of Smads 1/5/8 into the nucleus was greatest after 30 minutes and returned to baseline levels within 60 minutes [Figure 20B and 20C].

BMP-2 Stimulates Endothelial Cell Proliferation and Tube Formation

[00130]   The next study examined whether BMP-2 stimulates proliferation, tube

formation and/or migration of human endothelial cells. Unlike BMP-6, which stimulates

the migration of BAEC, BMP-2 did not induce the migration of HAEC [Figure 21A]. To

study the effects of BMP-2 on proliferation, the HAEC and HUVEC were treated with

recombinant BMP-2 for 24 hours and pulsed with H^3-thymidine for one hour. The effects

of BMP-2 on proliferation varied between the HAEC and HUVEC. BMP-2 stimulated a

dose responsive increase in DNA synthesis in HAEC [Figure 21B]. Relatively little

change in DNA synthesis was seen in the HUVEC following treatment with BMP-2

[Figure 21B]. There was no difference in proliferation when ^{3}H-thymidine incorporation

was measured 6 hours after adding BMP-2 to HUVEC. [Figure 21C]

[00131]   To study whether BMP-2 induced tube formation, the HAEC or HUVEC were

plated on Matrigel coated plates and treated with and without recombinant BMP-2

[Figure 22A-D]. BMP-2 produced a robust increase in the tube formation in both the

HAEC [Figure 22E] and HUVEC [Figure 22F]. The greatest effect on tube formation

occurred at 500 pg/ml of BMP-2 with a lesser effect at higher concentrations. Noggin

inhibited BMP-2 induced tube formation in both the HAEC [Figure 22E] and HUVEC

[Figure 22F].

BMP-2, but Not BMP-4, Is Aberrantly Expressed in Non-Small Cell Lung Carcinomas (NSCLC) and Stimulates Tumor Growth of A549 Cells In an Autocrine Manner

[00132] BMP-2 and BMP-4 are known to induce pluripotent cell differentiation, enhance cell migration, and stimulate proliferation during embryonic development. Despite being powerful morphogens, it was previously unknown whether BMP-2/4 have significant biological activity in human carcinomas or whether the mature active BMP-2/4 protein is aberrantly regulated and expressed in patient-derived tumors.

[00133] This aspect of the present invention reveals that the mature BMP-2 protein is highly over-expressed in human NSCLC with little to no expression in normal lung tissue or benign lung tumors. BMP-4 is expressed, but is not highly overexpressed. The expression of BMP-2 is localized specifically to the cancer cells. Recombinant BMP-2 stimulated *in vitro* the migration and invasiveness of the A549 and H7249 human lung cancer cell lines. *In vivo*, recombinant BMP-2 enhanced the growth of tumors formed from A549 cells injected subcutaneously into nude mice. Furthermore, inhibition of BMP-2 activity with either recombinant noggin or anti-BMP-2 antibody resulted in a significant reduction in tumor growth. This study shows that expression of the mature, active BMP-2 protein is disregulated in the majority of NSCLC. BMP-2 has important biological activity in lung carcinomas, as shown through its enhancement of tumor cell migration and invasion, as well as stimulating tumor growth *in vivo*.

[00134] The experiment used RDA to identify the expression of BMP-2 mRNA in a patient derived non-small cell lung carcinoma. High levels of expression of the mature active BMP-2 protein were shown in all of the NSCLC examined, with little to no

expression in normal lung tissue. This present study shows the mature BMP-2 protein is highly over-expressed in most or all patient-derived lung carcinomas, and that mature BMP-4 protein is not significantly expressed in human lung carcinomas.

[00135]   BMP-2 has important biological activity in human lung carcinomas. The natural BMP-2 inhibitor, noggin, caused a significant reduction of tumor growth of the A549 cells in nude mice. Noggin has a high affinity to BMP-2 and BMP-4 with a much lower affinity to BMP-7. Noggin has also been shown to inhibit growth differentiation factor-5 (GDF-5) and GDF-6. Although noggin could potentially inhibit other BMP proteins affecting tumor growth, the present invention shows that the inhibition was specific for BMP-2. The studies did not reveal expression of the mature BMP-4 protein in patient derived lung carcinomas or of BMP-7 or GDF-5 protein in human lung tumors. Furthermore, the findings that an anti-BMP-2 antibody also inhibited tumor growth and that recombinant BMP-2 enhanced tumor growth *in vivo* further support that BMP-2 has a significant role in lung tumorigenesis.

[00136]   The addition of recombinant BMP-2 to the A549 cells injected into nude mice did not induce bone or cartilage formation. The reason for this apparent discrepancy of expected BMP-2 *in vivo* activity is not known. However, the present invention further proves that not all biological activity associated with BMP-2/4 involves the formation of bone or cartilage. The biological response of BMP-2 or BMP-4 may depend not only on the particular cell types present, but may vary depending on the presence of other cytokines.

[00137] Since BMP-2 is a secreted protein, it can act in a paracrine and/or autocrine manner. This invention demonstrates that BMP-2 stimulates the migration of lung cancer cell lines *in vitro*. Therefore, one autocrine mechanism of BMP-2 is to enhance the invasiveness of a tumor by autocrine activation of tumor cells, which promotes their proliferation. [Figure 23] At high concentrations, BMP-2 suppressed the growth of the A549 cells in SFM, showing that it suppresses the growth of cancer cells. Since BMP-2 enhances tumor growth *in vivo*, this suggests that BMP-2 also acts by paracrine mechanisms. However, the effects of BMP-2 on cell growth may vary depending on the cell culture conditions. There are several potential mechanisms through which BMP-2 could enhance carcinogenesis in a paracrine manner, including inducing stem cell differentiation, enhancing migration of vascular smooth muscle cells and monocytes, and stimulating the production of an extracellular matrix.

[00138] Further supporting BMP-2's role in autocrine stimulation, the study also shows that BMP-2 is able to both stimulate and inhibit the proliferation of the same cancer cell line depending on the cell culture media. [Figure 23] This invention showed BMP-2 causes a significant decrease in the proliferation of A549 cells cultured in serum free media (SFM) over time. [Figure 23B] However, BMP-2 stimulated the proliferation of A549 cells in Dulbecco's Modified Eagle Medium (DMEM) fetal calf serum (FCS). [Figure 23C and 23D]

[00139] Consistent with BMP-2 being a growth promoting cytokine, BMP-2 induced a rapid increase in the expression of Id1 and cyclin E and phosphorylated Erk 1/2 and Rb. Surprisingly, the same pattern of expression of these genes was seen in both DMEM FCS

and SFM when examined at very early time points. However, when these same signaling

pathways were examined at a later time when the effects on proliferation between the two

cell culture conditions were clearly different, the expression of these genes was entirely

different. A549 cells treated with BMP-2 cultured in DMEM-FCS continued to show

signaling consistent with enhanced proliferation with phosphorylation of Erk 1/2 and Rb,

and a decrease in p21. While in SFM, there was a growth inhibitory pattern with

hypophosphorylation of Erk 1/2 and Rb, a decrease expression of cyclin E, and an

increased expression of p21. Together, these studies show that BMP-2 induces both

growth stimulating and growth inhibiting pathways but the growth inhibitory pathway is

blocked by undefined cytokines present in the DMEM FCS. Of interest is that BMP-2

induces signaling pathways in cancer cells known to promote tumorigenesis. BMP-2 has

not previously been shown to induce Erk signaling in cancer cells.

BMP-2 Induces Erk and Signaling Id1 in Cancer Cells

[00140]   The mechanism BMP-2 induces these signaling pathways is not known.

Because the activation of Smad 1/5 in SFM was not detected, this suggests that BMP-2

stimulates cyclin E, Id1, and phosphorylates Erk 1/2 and Rb by a Smad 1/5 independent

mechanism. Activated Erk 1/2 has been shown to induce the transcription of cyclin E.

Therefore, it is possible that BMP-2 induces phosphorylation of Erk 1/2 by a Smad 1/5

independent mechanism, which then causes an increase in cyclin E expression. Cyclin E

can then bind ckd-2 leading to Rb phosphorylation (inactivation) allowing for progression

through the cell cycle. BMP-2 also induces a rapid and strong activation of Id1 in A549

cells. The present study reveals that BMP-2 may also stimulate Id expression by Smad 1/5 independent mechanisms.

[00141] The study also found BMP-2 produced significantly more apoptosis in A549 cells cultured in SFM than in DMEM FCS. This may account for the differences in proliferation noted between the two cell culture conditions. Interestingly, BMP-2 has been shown to induce apoptosis in human osteoblasts which occurs by a Smad independent mechanism (42). However, BMP-2/4 has also been shown to inhibit apoptosis in mesenchymal stem cells and myocytes (43-44). Both of these studies showed that BMP-2/4 inhibited apoptosis through Smad 1/5 (43-44). These data suggest that in SFM, BMP-2 induced apoptosis because of the lack of active Smad 1/5, while in the presence DMEM FCS BMP-2 activated Smad 1/5 therefore inhibiting any apoptotic signaling, enhancing cell survival and proliferation.

[00142] In conclusion, these data suggest that BMP-2 is a growth promoting cytokine in cancer that involves the autocrine regulation of cancer cells. The biological response induced by BMP-2 is dependent on the cell culture conditions. In the presence of serum containing medium, BMP-2 stimulated a prolonged expression of signaling pathways known to be frequently dysregulated in lung cancer. These data further support the hypothesis that BMP-2 has an important role in promoting tumorigenesis.

EXAMPLES

Example 1: Identification of BMP-2 Using Representational Difference Analysis Subtraction Technique

[00143]   Representational difference analysis (RDA) subtraction technique was used to identify genes highly expressed in a non-small cell lung carcinoma obtained from a patient (tester) in comparison to normal bronchial human epithelial cells (driver). The technique for RDA described in the following references was followed: Holmes, M.L., et al., Molecular and Cellular Biology 19: 4182-4190 (1999); Hubank, M., Nucleic Acids Research 22:5640-5648 (1994). Normal human bronchial epithelial cells were purchased from Clonetics, BioWhitaker (Walkersville, Maryland) and were maintained in serum free media. Human tissue specimens were obtained directly from the operating room and immediately frozen in liquid nitrogen. Tissue was stored in liquid nitrogen at –70 °C.

[00144]   To perform RDA, mRNA was purified from the samples using Oligo dT columns (Pharmacia, Peapack, NJ) according to the manufacturer's instructions and cDNA was then obtained using the Pharmacia Time Saver cDNA synthesis kit also according to the manufacturer's instructions. cDNA was digested with Sau3A I endonuclease, R-linker ligated, and amplified by PCR. The R-linkers were removed and J-linkers ligated to the tester. The driver and tester cDNA were hybridized at 67 °C for 20 hours (driver in excess 100:1) and the subtracted tester cDNA amplified by PCR. A second round of subtraction was performed using N-linkers (driver in excess 800,000:1). The amplified PCR products were cloned into blue script and sequenced using an IBI Prism 377 DNA sequencer.

Known genes corresponding to the subtracted tumor cDNA were identified by a BLAST

database search.

Example 2: Detection of Expression of BMP-2 in Human Lung Cancer Specimens Using RT-PCR

[00145]   Reverse transcriptase polymerase chain reaction was performed using standard

techniques well known in the art. The forward primer was acgagagctctcactggtcc (SEQ ID

NO: 15). The reverse primer was cattccggattacatgaggg (SEQ ID NO: 16). The chain

reaction consisted of denaturation at 95° C for 1 min, annealing at 54°C for 1 min, and

extension at 72°C for 2 min for 33 cycles.

Example 3: Detection of Over-Expression of BMP and BMP Receptors in Various Cancer Tissue Specimens and Lung Cancer Cell Lines

[00146]   The inventor detected expression of BMP and BMP receptors in a number of

normal and cancerous tissue specimens and cells. As described above, all human tissue

specimens were obtained directly from the operating room and immediately frozen in

liquid nitrogen and stored at −70 °C. Normal human bronchial epithelial (NBE) cells

were purchased from Clonetics, BioWhitaker (Walkersville, Maryland) and were

maintained in serum free media. Immortalized human bronchial epithelial (IHBE),

BEAS-2B, cells were derived from normal bronchial epithelial cells immortalized with an

adenovirus-12-5-V40 hybrid virus. A549 and H7249 are highly invasive human lung

cancer cell lines. The cell lines were cultured in 5% fetal bovine serum (FBS) in

Dulbecco' s Modified Eagles medium (DME) containing penicillin, streptomycin, and

glutamine with 5% pCO_2 at 37°C. Western blot analysis was used to detect expression of

the BMP ligand and its receptors in all of these samples. Immunohistochemistry studies were performed to detect BMP in non-small cell lung carcinoma samples and normal lung tissue samples derived from patients.

Western Blot Analysis

[00147]   In preparation for Western blot analysis, cells were lysed in a modified RIPA buffer containing 150 ml NaC1, 50 ml Tris, pH 7.5, 1% NP 40, 10% deoxycholic acid, and protease inhibitor cocktail from Calbiochem. Tissue specimens were sonicated on ice in the same modified RIPA buffer. The protein concentration of the resulting samples was measured using the Bradford assay technique. Recombinant human BMP-2, purchased from R & D Systems and reconstituted in PBS with gelatin, served as a control. Total cellular protein of the samples and recombinant human BMP-2 were analyzed by SDS-PAGE, transferred to nitrocellulose filter (Schleicher and Schuell, Keene, NH) at 35 V for 16 hours at 4°C and then incubated with the desired primary antibody. Specific proteins were detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

[00148]   The primary antibodies that were used included mouse anti-human BMP-2, goat anti-human BMP-4, goat anti-human BMP-7, goat anti-human type IA BMP receptor, and goat anti-human type IB BMP-2 receptor. All of these antibodies, except the goat anti-human BMP-7 were purchased from R & D Systems in Minneapolis, MN. The goat anti-human BMP-7 antibody was obtained from Santa Cruz (Santa Cruz, CA).

Immunohistochemistry Analysis

[00149]　　To perform immunohistochemistry analysis, four micron Cryostat-cut sections were air dried before being fixed in cold acetone for 10 minutes. Sections were washed in cold 0.5 M PBS and intrinsic peroxidase was quenched with 0.03% periodic acid for 20 minutes at room temperature. Sections were then rinsed in cold PBS and 0.5% BSA/PBS was applied to the slides for 15 minutes in a humid chamber. Biotinylated BMP-2/4 (R & D Systems) was applied at a 1:25 dilution in 1% BSA/PBS and incubated overnight at 4°C. Two slides were run as negative controls. One slide was incubated with biotinylated BMP-2 preabsorbed with recombinant human BMP-2 at 1:10 Molar ratio. As a second negative control slide samples were incubated overnight at 4° C with normal rabbit serum. Slides were washed with cold PBS and incubated for one hour in Streptavidin horseradish peroxidase (Dako) at a 1:500 dilution in 1% BSA/PBS. Slides were then counterstained in 0.7% Toluidine Blue.

Example 4: Detection of Processing of Mature BMP-2 by Human Leukocytes

[00150]　　Cell culture media from the A549 cells was incubated with leukocytes isolated from whole blood for 16 hours. Then, a Western blot was performed, as described above, on the cell culture media. Mouse anti-human BMP-2 antibody (#MAB355, R & D Systems, Minneapolis, MN) was the primary antibody used to detect the C-terminal end of BMP-2. Goat anti-human BMP-2 (Research Diagnostics, Flanders, NJ) was used to detect the N-terminal end of BMP-2. A Western blot of the leukocytes was also

performed with an anti-furin primary antibody to determine that human leukocytes

express furin convertase.

**Example 5: Analysis of the Effect of BMP-2 and Noggin on Tumor Growth
and Tumor Vasculature In Vivo**

[00151]   Nude mice studies were conducted to determine the effect of BMP-2 and

noggin on tumor growth and tumor vasculature. 10^6 A549 cells were injected

subcutaneously into nude mice with Affi-Blue agarose beads coated with albumin,

recombinant human BMP-2, or recombinant mouse noggin. Both of these recombinant

proteins were purchased from R & D Systems and were reconstituted in PBS with gelatin.

Coating of Affi-Blue agarose beads with BMP-2 and noggin has been described in the

literature. (Abe, E., et al., J. Bone Miner Res. 15: 663-673 (2000); Tucker, A.S., et al.,

Science 282: 1136-1138 (1998); Zimmerman, L.B., et al., Cell 86: 599-606 (1996)) In

brief, 25 ug of Affi-blue agarose beads were incubated with 100 ug/ml albumin,

recombinant human BMP-2, or recombinant noggin for 2 hours and then washed 3 times

with PBS immediately prior to use. In separate experiments, the beads were not washed

prior to injection. The coated beads were injected with the A549 cells into nude mice

subcutaneously. To assess tumor growth after 12 or 19 days, the length, width, and depth

of the tumors were measured in mm. To assess tumor vasculature, tissue including a

tumor was harvested after six days. Gross observations of the tissue were made. In

addition, the tissue was stained with anti-CD31 antibody, which recognizes endothelial

cells. Vessels in five high power fields were counted by a person blinded to how the

tumors were created.

Example 6: Effect of BMP-2 and Noggin on VEGF and Sonic Hedgehog; Expression Western blot analysis of VEGF and Sonic Hedgehog in presence of BMP-2 and noggin

[00152]   Western blots, as described above, were performed on total cellular protein samples and cell culture media samples. The primary antibodies used to detect VEGF and sonic hedgehog were anti human VEGF from R & D Systems (Minneapolis, MN) and anti human sonic hedgehog from Santa Cruz (Santa Cruz, CA), respectively.

ELISA of VEGF in Presence of BMP-2 and Various Concentrations of Noggin

[00153]   The sandwich ELISA method was used to determine VEGF concentrations in the cell culture media of A549 cells treated with noggin and in the cell culture media of human aortic endothelial cells treated with BMP-2. 100 ul of the monoclonal capture antibody, diluted in carbonate buffer (sodium bicarbonate, sodium carbonate, pH 9.0), was added to each well of a MaxiSorb Nunc-Immuno plate and incubated overnight at 4°C. The plates were washed two times with washing buffer (1x PBS with 0.0005% tween-20). Then, 200 ul of blocking buffer (1x PBS with 1% BSA) was added per well and incubated for 2 hours at room temperature. The plates were then washed 4 times with washing buffer.

[00154]   The recombinant protein standards and samples (100 ul per well) were added and the plate was then incubated overnight at 4°C. The plates were washed 5 times with washing buffer. The biotinylated detection antibody was diluted in incubation buffer (1x PBS with 10% fetal bovine serum) for a final concentration of 1 ug/ml. 100 ul of the detection antibody was added per well and incubated for 1 hour on a shaker at room

temperature. The plates were washed 6 times with washing buffer and 100 ul of

streptavidin-HRP conjugate (1:10,000) was added per well. The plates were incubated

for 45 minutes at room temperature on a shaker and then washed 6 times with washing

buffer. 100 µl/well of the substrate reagent (0.2 M citrate buffer, 1 mg/ml o-

phenylenediamine dihydrocholoride (OPG), 3% hydrogen peroxide) was added and

covered with aluminum foil for ten minutes. The reaction was stopped with 100 µl/well

of 2M sulfuric acid and absorbance determined using an automated plate reader with a

490/690 filter. The protein concentration was then determined from the standard curve.

**Example 7: Identification of BMP-2 as a Stimulant of Human Lung Cancer
Cell Migration and Invasion**

Migration Assay In Monolayer Culture

[00155]   To detect BMP-induced migration in a monolayer culture, recombinant human

BMP-2 (R & D systems, Minneapolis, MN) was coated to Affi-Blue agarose beads (Bio

Rad, Hercules, CA) as described in the literature. (Vainio, S.; et al., Cell 75: 45-58

(1993); Sloan, A.J., et al., Arch Oral Biol. 44: 149-156 (1999)) Briefly, 100 ml of the

Affi-Blue agarose beads were incubated with either 10 ml of recombinant BMP-2

reconstituted in PBS with gelatin (100 mg/ml) or PBS alone at 37°C for 2 hours, washed

with PBS, and reconstituted with 40 ml of PBS. Glass cover slips were coated with

serum free media containing BSA, fibronectin and collagen and 50,000 cells were plated

per cover slip in serum free media. Two microliters of the Affi-Blue agarose beads

coated with recombinant BMP-2 or dilution buffer were placed in linear fashion next to

the cover slips.

Chemotactic Assay

[00156]   In the chemotactic assay, fifty thousand cells were placed in the upper chamber of an 8 micron transwell migration chamber (Becton Dickinson, Bedford, MA) and 300 ml of serum free media with 0 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml recombinant human BMP-2 placed in the lower well. After 24 hours, the filters were then removed and the top of the filter wiped with a cotton swab and the cells that migrated through the filters were stained with Syto-16 intercalating dye. Five high power fields were counted using fluorescent microscopy. To show that noggin inhibits BMP-2 induced migration, the experiment was also performed with each of the following in the lower well of the transwell chamber: media alone, recombinant BMP-2 (500 ng/ml), and noggin (10 ug/ml) with recombinant BMP-2 (500 ng/ml).

Matrigel Invasion Assay

[00157]   Invasion was studied using transwell chambers coated with 100 ml of Matrigel (Becton Dickinson). Fifty thousand cells were placed in the upper chamber and 300 ml of serum free media with 0 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml recombinant BMP-2 placed in the lower wells. After 48 hours, the Matrigel was removed and cells that had migrated through the filter were stained with Syto-16 intercalating dye and 5 high power fields counted using fluorescent microscopy.

Example 8: BMP-2, but not BMP-4, is Overexpressed in NSCLC and Stimulates Tumor Growth

Cell Lines

[00158] The immortalized normal human bronchial epithelial cells (BEAS-2B) and human lung cancer cell lines A549 and H7249 were cultured in Dulbecco's Modified Eagles medium (DME) with 5% fetal bovine serum (FBS) containing 1% penicillin/streptomycin, and 1% glutamine. These cells were obtained and preserved as described in Example 3.

Antibodies and Recombinant Proteins

[00159] Monoclonal anti-human BMP-2 antibodies MAB355 and MAB3551 (R & D Systems, Minneapolis, MN) were used to detect BMP-2 by Western blot analysis. Goat anti-human BMP-2/4 (R & D Systems) was used for immunohistochemistry. Goat anti-human type IA, IB, and II BMP receptor antibodies, mouse IgG antibody, and anti-human BMP-4 monoclonal antibody were obtained from R & D Systems. A goat anti-BMP-4 antibody was obtained from Santa Cruz (Santa Cruz, CA). Both anti-BMP-4 antibodies were used to assess BMP-4 expression by Western blot analysis. Recombinant human BMP-2, recombinant human BMP-4, and recombinant noggin were purchased from R & D Systems. Recombinant proteins were reconstituted in phosphate buffered saline (PBS) with 0.1% gelatin. An anti-BMP-2 monoclonal antibody, which was a gift from the Genetics Institute (Cambridge, MA), was co-injected with the A549 cells into female NCJ nude mice.

Migration Assay

[00160]   To examine whether BMP-2 enhanced the migration of lung cancer cells fifty

thousand A549 or H7249 cells were placed in the upper chamber of an 8-micron transwell

migration chamber (Becton Dickinson, Bedford, MA). In the lower well was 300 µl of

LHC serum free media (BioFluids, Rockville, MD) containing recombinant human

BMP-2 (1, 10, 100, or 500 ng/ml) or an equal volume of PBS with 0.1% gelatin. After 24

hours, the filters were removed and the top of the filter was wiped with a cotton swab.

Cells that had migrated through the filters were stained with Syto-16 intercalating dye

(Molecular Probes, Eugene, OR). Five high power fields were counted using fluorescent

microscopy. To inhibit BMP-2, 500 ng/ml of recombinant BMP-2 was incubated with 10

µg/ml of recombinant noggin in serum free media at 37˚C for one hour before placing the

BMP-2 into the lower chamber.

Migration Assay In Monolayer Culture

[00161]   To assess whether BMP-2 stimulates the migration of tumor cells growing in

monolayer culture, it was examined whether A549 and H7249 cells growing on glass

cover slips migrated toward Affi-Blue beads coated with recombinant human BMP-2.

Recombinant human BMP-2 (R & D systems, Minneapolis, MN) was coated with Affi-

Blue agarose beads (Bio Rad) as previously described. Briefly, 100 µl of the Affi-Blue

agarose beads were incubated with 10 µl of BMP-2 (100 µg/ml) or PBS with 0.1% gelatin

at 37ºC for 2 hours. The beads were then washed with PBS and reconstituted with 40 µl

of PBS. Glass cover slips were coated with serum free media containing BSA,

fibronectin and collagen and 50,000 cells were plated per cover slip. After allowing the

cells to attach for 12 hours the cover slips were placed into a 6 well plate containing

serum free media. Two microliters of the Affi-Blue agarose beads coated with

recombinant BMP-2 or dilution buffer were placed in linear fashion next to the cover

slips. Five days later the cells, which migrated off the cover slips, were photographed.

Invasion Assay

[00162]   The next issue was whether BMP-2 enhanced the invasiveness of tumor cells

by determining whether recombinant BMP-2 stimulated the migration of A549 and

H7249 cells through the extracellular tumor matrix, Matrigel (Becton Dickinson,

Bedford, MA). 100 µl of Matrigel was placed in the upper well of an 8-micron transwell

migration chamber. Fifty-thousand A549 or H7249 cells were placed in the upper

chamber and 300 µl of LHC serum free media supplemented with recombinant human

BMP-2 (1, 10, 100, or 500 ng/ml) or an equal volume of PBS with 0.1% gelatin was

added to the lower well. After 48 hours, the Matrigel the cells on the upper side of the

filter were removed using a cotton swab. Cells that migrated through the filter were

stained with Syto-16 nuclear dye and examined by fluorescent microscopy.

Growth Assay

[00163]   To determine the effects of BMP-2 on the monolayer growth of the A549 cells,

seventy-five thousand cells were plated onto 6 well tissue culture plates containing LHC

serum free media. After allowing the cells to adhere for 12 hours they were treated with

recombinant BMP-2 or with vehicle alone (PBS with 1% gelatin). Two days later cells

were detached and counted using a hemacytometer.

Western Blot Analysis

[00164]   Tissue specimens in a modified RIPA buffer were briefly sonicated on ice.

Protein concentration was measured using the Bradford assay. Protein samples were

prepared under reducing conditions. Total cellular protein was analyzed by a 15% SDS-

PAGE, transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) at 35 V for 16

hours at 4°C. The blots were then incubated overnight at 4°C with the appropriate primary

antibody in Tris-buffered Saline with 1% Tween (TBST) and 5% nonfat dried milk.

Specific proteins were detected using the enhanced chemiluminescence system

(Amersham, Arlington Heights, IL). Relative BMP-2 expression was analyzed using NIH

Imaging. Pixel density of the mature BMP-2 protein and actin were determined on each

of the developed immunoblots. A relative actin value was determined in each sample by

dividing its pixel density by the pixel density from the sample with the highest expression

of actin. The sample with the highest actin level on the blot was given a value of 1 and

the remaining samples as a fraction of that value. The relative BMP-2 expression was

determined by dividing the BMP-2 pixel density by its relative actin value.

Northern Blot Analysis

[00165]   The m-RNA obtained from the driver and tester was size fractionated on a 1%

agarose-formaldehyde gel in a MOPS (0.2M 3-N-morpholino-propanesulfonic

acid/0.05M Na Acetate/0.01M EDTA) buffer. The m-RNA was transferred to a

nitrocellulose membrane by capillary transfer. The m-RNA was cross-linked to the

membrane using ultraviolet light. The subtracted BMP-2/4 cDNA was radiolabeled with

P^{32} using the All-In-One Random Priming mix (Sigma, St. Louis, MO). The probe was

denatured by boiling and incubated with the blots in PerfectHyb Plus hybridization buffer

(Sigma) for 12 hours. Membranes were washed in high stringent conditions and exposed

to Kodak XAR film with an intensifying screen.

RT-PCR

[00166]   Total RNA was extracted from a patient derived tissue sample using Trizol

(Gibco, Rockville, MD). First strand cDNA was synthesized using the Advantage for

PCR kit (Clontec, Palo Alto, CA) following the manufacturer's instructions. BMP-2

cDNA was amplified using primers (F) 5'-cct gag cga gtt cga gtt g-3' [SEQ ID NO: 17],

and (R) 5'-cac tcg ttt ctg gta gtt c-3' [SEQ ID NO. 18] at 95° C for 1 minute, 50° C for 1

minute, 72° C for 2 minutes for 30 cycles. The expected size of the amplified BMP-2 was

230 base pairs. BMP-4 was amplified using primers (F) 5'-tac ctg aga cgg gaa gaa a-3'

[SEQ ID NO: 19] and (R) 5'- cca gac tga agc cgg taa ag-3' [SEQ ID NO: 20] at 95° C for

1 minute, 56° C for 1 minute, 72° C for 2 minutes for 33 cycles. The expected size of the

amplified BMP-4 was 211 base pairs. The amplified bands were gel purified and

sequenced at the core UMDNJ-RWJMS sequencing facility using an IBI Prism 377 DNA

sequencer.

Cloning and Transfection of BMP-2

[00167]   Full-length human BMP-2 cDNA (gift from Genetics Institute) was cloned into

the pcDNA3.1 expression vector (Invitrogen) in both the sense (BMP-2-S) and anti-sense

(BMP-2-AS) directions. A549 cells with forced expression of BMP-2-S were also co-

transfected with a pCMV-EGFP vector (BD Biosciences, Palo Alto, CA) which

constitutively expresses green fluorescent protein (GFP). Transfection was performed by

electroporation using 30 µg of BMP-2-S and 10 µg of GFP at 0.975 µF capacitance and

0.2 Kv. Control cells were transfected with the pcDNA3.1 and the GFP expression vector

(A549-GFP). Transfected cells were cultured in 5% FCS DME media containing 50

µg/ml of neomycin. After expanding the transfected A549 cells in selective medium,

cells expressing GFP were bulk sorted using flow cytometry. To obtain A549 cells

expressing BMP-2 in the anti-sense direction, A549 cells were transfected with BMP-2-

AS or pcDNA3.1 vector (A549-V) by electroporation. These cells were not co-

transfected with the GFP expression vector and transfected cells were obtained by

culturing in selective media. BMP-2-AS and A549-V sub-clones were obtained by

limiting dilution in 96 well plates placing 1 cell for every 5 wells. Seven sub-clones of

BMP-2-AS and A549-V were then selected.

Immunohistochemistry

[00168]   Tissue samples were placed in Optimal Cutting Temperature (OCT) and snap

frozen in liquid nitrogen. Four micron Cryostat sections were air dried before being fixed

in cold acetone for 10 minutes. Sections were washed in cold 0.5M PBS and intrinsic

peroxidase was quenched with 0.03% periodic acid for 20 minutes at room temperature. Sections were then rinsed in cold PBS and 0.5% BSA/PBS was applied to the slides for 15 minutes in a humidified chamber. Biotynilated polyclonal BMP-2/4 antibody (R&D Systems) was applied at a concentration of 2 ug/ml in 1% BSA/PBS and incubated overnight at 4°C.

[00169]   Negative controls were slides in which the samples were incubated overnight at 4°C with normal rabbit serum without primary antibody. In addition, we performed a competition assay by incubating the biotynilated BMP-2/4 antibody with recombinant human BMP-2 at a 1:10 Molar ratio for 2 hours before the overnight incubation. Following the overnight incubations the slides were washed with cold PBS and incubated for one hour in Streptavidin horseradish peroxidase (Dako) at a 1:500 dilution in 1% BSA/PBS. Slides were then counterstained in 0.7% Toluidine Blue.

Effect Of BMP-2 On Tumor Growth In Nude Mice Studies

[00170]   To assess the effects of BMP-2 on tumor growth *in vivo,* A549 cells were co-injected subcutaneously into female NCJ athymic nude mice with recombinant BMP-2 or the BMP-2 antagonist noggin. Recombinant protein was delivered to the tumors using Affi-Blue agarose beads as previously described. In brief, 25 μg of Affi-Blue agarose beads were incubated with 20 μl of 100 μg/ml of BSA, recombinant human BMP-2, or noggin for 2 hours, and then washed 3 times with PBS immediately prior to use. In separate experiments, the beads were not washed prior to injection. The coated Affi-Blue agarose beads were co-injected with the 10^7 A549 cells subcutaneously into the flanks of

NCJ nude mice. In a separate study 10^7 A549 cells were co-injected with 20 μg of an anti-BMP-2 monoclonal antibody reported to inhibit its activity. As a control, A549 cells were co-injected with 20 μg of an isotype control antibody. Fourteen to 19 days following injection the animals were sacrificed and the tumors were removed and measured in 3 dimensions (length x width x depth). The mice studies were approved by the Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

Statistical Analysis

[00171]   Assessment of recombinant BMP-2 protein and recombinant Noggin on tumor growth in athymic nude mice was analyzed from 5 independent experiments. *In vivo* studies using an anti-BMP-2 antibody were performed twice. All other experiments were performed at least 3 times. The size of tumors formed from A549 cells treated with recombinant BMP-2, recombinant noggin, or anti-BMP-2 antibody is reported as the mean + SEM percentage of tumors formed from controls. Results were evaluated by one-way ANOVA using the Student-Newman-Keuls procedure for adjustment of multiple pairwise comparisons between treatment groups. Differences with P values < .05 were considered statistically significant.

Representational Difference Analysis (RDA)

[00172]   The RDA subtraction technique was performed to identify m-RNA that is uniquely or highly expressed in human lung carcinomas in comparison to normal bronchial epithelium. cDNA from immortalized human bronchial epithelial cells (IHBE) was hybridized in excess (driver) to cDNA obtained from a patient derived NSCLC

(tester). The known genes identified by RDA were alpha-1-antitrypsin, cytokeratin 6, and

BMP-2/4. It was not determined whether the sequenced cDNA was BMP-2 or BMP-4

because the amplified region (b.p. 766 to 863) is conserved in both. Alpha-1-antitrypsin

has previously been reported to be over-expressed in 87% of human lung carcinomas.

Since BMP-2/4 are powerful morphogens that have not been fully characterized in

cancer, their expression and biological activity was further examined in human lung

carcinomas.

BMP-2/4 mRNA Expression In Human Lung Carcinomas

[00173]   The BMP-2/4 cDNA obtained from the RDA was labeled with P^{32} and

hybridized to a Northern blot containing the original m-RNA from the IHBE cells (driver)

and NSCLC (tester). This blot revealed high expression of the BMP-2/4 mRNA in the

tumor, with no expression found in the IHBE cells. RT-PCR confirmed that BMP-2

mRNA was not expressed in the IHBE. BMP-2 expression was identified in the A549

human lung cancer cell line. However, BMP-4 expression was detected by PCR in the

IHBE cells. Next, it was examined whether the BMP-2 and BMP-4 m-RNA was

expressed in other NSCLC. All 4 of the human lung tumors examined by RT-PCR

demonstrated BMP-2 and BMP-4 expression. Sequencing of the PCR product confirmed

the primers were specific for BMP-2 or BMP-4.

The Mature BMP-2 Protein Is Highly Over-Expressed In Human Lung Carcinomas

[00174] The BMP-2 and BMP-4 proteins are translated as precursor proteins and must be cleaved by a proprotein convertase to produce a mature active peptide. The next study examined whether the mature BMP-2 and/or BMP-4 proteins are expressed in patient derived lung carcinomas. Immunoblots probed with a monoclonal BMP-2 antibody showed high expression of a 14 kD mature BMP-2 protein in 11 of 12 NSCLC examined. In comparison, little to no expression of the mature BMP-2 protein in 10 normal lung tissue specimens was found. When the level of BMP-2 expression was assessed relative to the level of actin by quantitative scanning, BMP-2 expression was found to be 26 times higher in the NSCLC than that of normal lung tissue. Expression of BMP-2 was not increased in benign interstitial lung disease or a benign lung tumor (hamartoma). Recombinant human BMP-2 served as a positive control. BMP-2 was also found to be expressed in the A549 and H7249 human lung cancer cell lines. The bone forming osteosarcomas have previously been reported to express BMP-2/4 by immunohistochemistry. As expected, the SoAS osteosarcoma cell line also expressed a 14 kD mature BMP-2 protein.

[00175] It was additionally discovered that the monoclonal anti-BMP-2 antibody (MAB355) used in the above experiments cross reacts with recombinant BMP-4 protein. Therefore, the study probed the immunoblots with a second monoclonal anti-BMP-2 antibody (MAB3551), which is reported to be specific for BMP-2. The studies confirmed that this antibody does not recognize recombinant BMP-4 protein. The MAB3551

antibody also showed that the mature BMP-2 is over-expressed in NSCLC. The

MAB3551 anti-BMP-2 antibody recognized predominately a 17 kD mature BMP-2

protein, but also demonstrated expression of a 14 kD mature protein in NSCLC.

[00176] Difference in the expression pattern between the two antibodies can be

explained by the immunogens used to produce these anti-BMP-2 antibodies. The

MAB355 antibody was produced against an Escherichia coli derived mature human

BMP-2 protein (non-glycosylated). The MAB3551 monoclonal antibody was raised

against a glycosylated mature human BMP-2 protein (NSO derived recombinant human

BMP-2). Although the MAB355 antibody recognized predominately a 14 kD protein,

expression of a 17 kD protein on some of the immunoblots was found. The predicted size

of BMP-2 under reducing conditions is 14 kD, however, following glycosylation BMP-2

is reported to be 17 kD. These data suggest that lung carcinomas produce predominately

a 14 kD non-glycosylated BMP-2 protein. Prior studies have shown that glycosylated and

non-glycosylated mature BMP-2 proteins have equivalent biological activity, *in vivo*.

[00177] Next examined was whether the mature active BMP-4 protein is expressed in

human lung tumors. No detectable expression of the mature BMP-4 protein could be

seen in either malignant or normal lung tissue when probing immunoblots with a BMP-4

specific monoclonal antibody. Using a second antibody specific for BMP-4, was still

undetectable expression of the mature BMP-4 protein. To verify further the MAB355

anti-BMP-2 antibodies recognized the native mature BMP-2 protein, full length human

BMP-2 cDNA was transfected into the A549 cells (BMP-2-S). The cell culture media

was obtained from the BMP-2-S and vector control A549 cells and Western blot analysis

performed. Immunoblots probed with the MAB355 anti-BMP-2 antibody showed higher

expression of the 14 kD BMP-2 protein in the BMP-2S cells compared to controls. In

addition, BMP-2 cDNA was transfected in the anti-sense direction into the A549 cells.

All 7 subclones containing the anti-sense BMP-2 cDNA had a lower expression of the 14

kD protein on immunoblots probed in comparison to the 7 subclones transfected with

insertless vector. Together these data demonstrate that the mature BMP-2, but not the

mature BMP-4 protein, is highly over-expressed in human lung carcinomas.

[00178]   To help determine which cells are expressing BMP-2 in the NSCLC, BMP-2

expression in a NSCLC by immunohistochemistry was examined. BMP-2 expression was

detected in the cytoplasm of the tumor cells. BMP-2 was not found to be expressed in

normal lung tissue or surrounding stromal cells. To confirm the antibody recognized

BMP-2, the antibody was incubated with recombinant human BMP-2 prior to

immunostaining. This led to a complete inhibition of the BMP-2 signal in the tumor

specimen.

Expression of BMP-2 Receptors In Human Lung Carcinomas

[00179]   BMP-2 induces a physiological response through the activation of receptors

specific for the BMPs. Intracellular activation begins with BMP-2 binding to either type

IA or IB BMP receptors, which leads to the binding of this complex to the type II BMP

receptor. The type II receptor phosphorylates the type I receptor, which then directly

phosphorylates the Smad transcription factors. It was found that the BMP type IA, IB,

and II receptors are expressed in both primary lung tumors and normal lung tissue.

However, in contrast to the high level of expression of the BMP-2 ligand in primary lung cancers, the same level of expression of the type IA receptor between the lung carcinomas and normal lung tissue was found. The expression of the type IB and type II receptors was lower in the lung tumor than that of normal lung tissue. The IHBE, A549 and H7249 cells were also found to express type IA, IB and type II receptors. These data suggests that both cancer cells and normal lung tissue have the potential capability of being activated by the BMP-2 ligand.

BMP-2 Stimulates the Migration and Invasiveness of Human Lung Cancer Cells

[00180]   BMP-2 and BMP-4 have been shown to stimulate the migration of non-cancerous human cells. Since migration is important for tumors to invade and metastasize, it was examined whether recombinant BMP-2 stimulates the migration of the A549 and H7249 human lung cancer cell lines *in vitro*. When recombinant BMP-2 was placed in the lower well of a migration chamber, it caused a dose responsive increase in the migration of both the A549 and H7249 cells. The BMP-2 antagonist, noggin, completely inhibited the ability of BMP-2 to stimulate the migration of the A549 cells. Next, it was determined whether BMP-2 enhanced the migration of the lung cancer cell lines growing in monolayer cell culture. The A549 and H7249 cells were cultured on glass cover slips and placed in 6 well plates containing Affi-Blue agarose beads coated with PBS with 0.1% gelatin or recombinant BMP-2. After 5 days, the number of cells that had migrated off the cover slips and were growing in the six well plate was assessed. There was only an occasional cluster of A549 or H7249 cells growing on the six well

plates when cultured with control beads. However, when A549 and H7249 were cultured with Affi-Blue agarose beads coated with recombinant BMP-2, there was consistently a large number cells that migrated off the glass cover slips and were growing on the six well plate.

[00181] BMP-2 stimulation of the migration of the A549 and H7249 lung cancer cells through the tumor matrix Matrigel was examined to assess whether BMP-2 may enhance the invasiveness of cancer cells. Recombinant BMP-2 again produced a dose responsive increase in the migration of both the A549 and H7249 cells through the Matrigel coated chambers.

In Vitro Growth Effects of BMP-2 on The A549 Cells

[00182] Next assessed were the effects of recombinant BMP-2 on the growth of the A549 cells *in vitro*. It was found that recombinant BMP-2 caused only minimal growth suppression of the A549 cells. In serum free conditions, BMP-2 at a 100 ng/ml caused a 15% decrease in growth of the A549 cells after 2 days. Lower concentrations of BMP-2 had no effect on the growth of the A549 cells.

BMP-2 Enhances *In Vivo* Tumor Growth

[00183] Since BMP-2 is a secreted protein, it was hypothesized that it may effect the growth of the A549 cells differently *in vivo* than it does *in vitro*. To answer this question, required assessment of tumor growth of the A549 cells in athymic nude mice treated with recombinant BMP-2 or by inhibiting BMP-2 activity with noggin or an anti-BMP-2 antibody. Noggin coated Affi-Blue agarose beads were co-injected with the A549 cells

subcutaneously into nude mice. Noggin has a high binding affinity for BMP-2 and BMP-4 preventing their activation of the BMP receptors. . Noggin treated 549 cells (n=14) consistently formed tumors which were less than half the size of A549 cells treated with albumin (n=15). Co-injection of A549 cells with an anti-BMP-2 monoclonal antibody previously reported to inhibit BMP-2- induced migration of smooth muscle cells, (n=4) also produced an approximately 60% reduction in tumor growth when compared to mice co-injected with a control antibody (n=4).

[00184] A549 cells co-injected into nude mice with Affi-Blue agarose beads coated with recombinant human BMP-2 (n=15) formed tumors which were approximately 50% larger than that of A549 cells treated with BSA (n=15). Tumors were stained with hematoxylin and eosin and examined by a surgical pathologist for the presence of bone and/or cartilage. There was no evidence of bone and/or cartilage in any of the tumor formed from A549 cells treated with recombinant BMP-2. Together these data indicate that BMP-2 produced from the A549 cells enhances tumor growth *in vivo*, which is not associated with the formation of bone.

Example 9: BMP-2 Induces Proliferation of Lung Cancer Cells in an Autocrine Manner

Cell Lines and Acquisition of Tissue Specimens

[00185]   The immortalized normal human lung cancer cell line A549 were cultured in Dulbecco's Modified Eagles medium (DME) (Sigma Aldrich, St. Louis, MO) with 5% fetal bovine serum (FBS) containing 1% penicillin/streptomycin, and 1% glutamine. Cells were kept in a humidified incubator with 5% CO_2 at 37 °C.

Antibodies and Recombinant Proteins

[00186]   Primary antibodies against phosphorylated Smad 1 (Upstate Biotechnology,

Lake Placid, NY), Id1 (Santa Cruz, Santa Cruz, CA), cyclin E (Santa Cruz)

phosphorylated ERK-1/2 (Promega, Madison, WI), Actin (Sigma, St. Louis, MO) were

used for Western blot analysis. Antibody recognizing Smad 1, 5, and/or 8 (Santa Cruz,

Santa Cruz, CA) was used in immunoblots and immunofluorescence studies.

Recombinant human BMP-2 was purchased from R & D Systems. Recombinant proteins

were reconstituted in phosphate buffered saline (PBS) with 0.1% gelatin.

Western Blot Analysis

[00187]   Total cellular protein was obtained using RIPA buffer. Protein concentration

was measured using the Bradford assay. Protein samples were prepared under reducing

conditions. Total cellular protein was analyzed by a 5%-15% SDS-PAGE, transferred to

nitrocellulose (Schleicher and Schuell, Keene, NH) at 35 V for 16 hours at 4 °C. The

blots were then incubated overnight at 4 °C with the appropriate primary antibody in

either Tris-buffered Saline with 1% Tween (TBST) and 5% nonfat dried milk or

Membrane Blocking Solution (Zymed, San Francisco, CA). Specific proteins were

detected using the enhanced chemiluminescence system (Amersham, Arlington Heights,

IL).

Proliferation Assay

[00188]   A549 cells were plated at 2.0 x 10^5 cells/well in 6 well tissue culture plates

(Falcon, Franklin Lakes, NJ) in DMEM supplemented with 5% FCS and allowed to

adhere overnight. Experiments were then performed in serum free media (SFM) (LHC-8,

Biofluids, Rockville, MD) or DMEM 5% FCS. Cells were treated with 1, 10, or 100

ng/ml BMP-2 or 4mM HCl/0.1%BSA (vehicle control) and for either 6 hours or 24 hours.

In brief, cells were incubated with 4 °Ci/ml H^3 thymidine at 37 °C for 1 hour. Cells were

washed twice with ice cold PBS and fixed with 5% trichloroacetic acid for 10 minutes.

Cells were then washed with 70% ethanol, lysed with 1%SDS/10mM NaOH and

incorporated H^3 thymidine measured in a scintillation counter. The results of this

experiment are shown in Figure 24.

Smad Localization

[00189]   A549 cells were grown on glass cover slips were then cultured in DMEM 5%

FCS or SFM. A549 cells were then treated with 10ng/ml BMP-2 for 0, 15, 30, 45, and 60

minutes. Cells were fixed with 3.7% formaldehyde, permealized with 0.5% Triton X, and

blocked with 1% BSA / PBS. Cells were incubated overnight with a 1:50 dilution of anti-

Smad 1/5/8 antibody at 4 °C and AlexaFluor 488 anti-goat was used as the secondary

antibody (Molecular Probes, Eugene, OR). To inhibit BMP-2, 40 ng of recombinant

noggin was added to the BMP-2 treated cells. Negative controls included primary

antibody incubated with blocking peptide (Santa Cruz) and AlexaFluor 488 without

primary antibody. Smad 1/5/8 localization was assessed by immunofluorescent

microscopy.

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In Vivo Tumor Growth

[00190]   To assess the effects of BMP-2 on tumor growth *in vivo* A549 cells were coinjected subcutaneously into female NCJ athymic nude mice with recombinant BMP-2 or the BMP-2 antagonist, noggin. Recombinant protein was delivered to the tumors using Affi-Blue agarose beads as previously described. 25 µg of Affi-Blue agarose beads were coated with 20 µl of 100 µg/ml of albumin, or recombinant human BMP-2. The coated Affi-Blue agarose beads were co-injected with the 10^7 A549 cells subcutaneously into the flanks of NCJ nude mice. Fourteen to 19 days following injection the animals were sacrificed and the tumors were snapped frozen in liquid nitrogen and protein collected for Western blot analysis. A section of the tumor was also placed in Optimal Cutting Temperature (OCT) then frozen for immunohistochemistry studies.

Apoptosis Studies—Immunohistochemistry

[00191]   Frozen samples were cut with a dermatome to obtain 5 micron sections. The samples were incubated with a rat anti-Ki-67 antibody at a 1:20 dilution overnight at 4 °C. The anti-rat IgG was incubated at a 1:300 dilution at room temperature for 20 minutes. Positive control included a mouse liver. Negative controls consisted of samples treated with secondary antibody alone.

[00192]   In brief, four micron sections were cut using a dermatome and placed on slides. Each sample was stained for Ki-67 using the M.O.M. kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The slides were blocked for 1 hour in blocking reagent and mouse anti-human Ki-67 monoclonal antibody was diluted

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to 1:100 in the blocking reagent and incubated overnight at 4 °C. Each section was

incubated with secondary antibody (1:250) for 10 minutes and then with Vectastain Elite

ABC reagent as a tertiary conjugate for 5 minutes. DAB chromagen (DAKO,

Carpenteria, CA) was used as the final stain and toluidine blue used for background

staining. The graphical results of the cell growth with BMP-2 as compared to controls

when examined in a 40X field are shown in Figure 25. A photograph of the cell death in

control cells as compared to the much more active cell growth in the BMP-2 treated cells

is shown in companion Figure 26.

Apoptosis Studies

[00193]   A549 cells were plated onto 35mm cell culture dishes at a density of 6x105 in

DMEM supplemented with 5% FCS and allowed to adhere overnight. Cells were then

cultured in DMEM 5% FCS or SFM and treated with 10ng/ml or 100ng/ml rBMP-24 for

hours. Apoptosis was examined using JC-1 Mitochondrial Potential Sensor (Molecular

Probes, Eugene, OR) and by assessing nuclear condensation using ethidium bromide

staining. JC-1 is a fluorescent dye, which specifically targets mitochondrial membranes.

JC-1 has been used to detect membrane depolarization, which occurs during apoptosis.

When the mitochondria are polarized the JC-1 forms J-aggregates, which become

fluorescent red.

[00194]   When the mitochondria are depolarized, JC-1 it does not accumulate in the

nucleus and fluoresces green. JC-1 is added to live cells at 5g/ml and then washed in

PBS. The cells were placed the medium to incubate for 15 minutes in cell culture

conditions. Cells were protected from light and washed twice with warmed 1X PBS and immediately observed under fluorescence to assay the apoptotic cell to live cell ratio.

Statistical Analysis

[00195]   Assessment of recombinant BMP-2 protein and recombinant Noggin on tumor growth in athymic nude mice was analyzed from 5 independent experiments. In vivo studies using an anti-BMP-2 antibody were performed twice. All other experiments were performed at least 3 times. The size of tumors formed from A549 cells treated with recombinant BMP-2, recombinant noggin, or anti-BMP-2 antibody is reported as the mean + SEM percentage of tumors formed from controls. Results were evaluated by one-way ANOVA using the Student-Newman-Keuls procedure for adjustment of multiple pairwise comparisons between treatment groups. Differences with P values < .05 were considered statistically significant.

Example 10: BMP-2 Enhances Neovascularization of Developing Tumors Cell Culture and Reagents

[00196]   HAEC and HUVEC were obtained from Bio-Whittaker (Walkersville, MD). Endothelial cells were maintained in EBM-2 supplemented with 2% FCS and growth factors supplied in the bullet kit (Bio-Whittaker). The A549, A549-AS, and A549-vector lung cancer cell lines were prepared and maintained. Earlier recombinant BMP-2, noggin, and VEGF were purchased from R & D Systems (Minneapolis, MN). Primary antibodies against BMPR IA, BMPR IB, BMPR II (R & D Systems), phosphorylated Smad 1 (Upstate Biotechnology, Lake Placid, NY), Id1 (Santa Cruz, Santa Cruz, CA),

phosphorylated ERK-1/2 (Promega, Madison, WI), Actin (Sigma, St. Louis, MO) were used for Western blot analysis. Antibody recognizing Smad 1, 5, and/or 8 was used in immunofluorescence and immunoprecipitation studies (Santa Cruz, Santa Cruz, CA) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) for immunohistochemistry (BD Pharmingen, San Diego, CA).

Migration Assay

[00197] 3.0×104 HAEC cells in EBM-2 supplemented with 0.5% FCS and growth factors excluding VEGF (Bio-Whittaker) were placed in the upper chamber of a transwell migration chamber (Becton Dickinson, Bedford, MA). Medium containing 1ng/ml BMP-2, 50ng/ml VEGF, or 4mM HCl /.1%BSA (vehicle control) was added to the lower chamber. After 24 hours the cells that had migrated were stained with Giemsa (Sigma) and ten 40X fields were counted.

Proliferation Assay

[00198]   HAEC or HUVEC cells were plated at 2.0×105 cells/well in 6 well tissue culture plates (Falcon, Franklin Lakes, NJ) in EBM–2 with 2% FCS. Cells were treated with 1, 10, or 100 ng/ml BMP-2 or vehicle control for 24 hours then pulsed with 4 Ci/ml 3H -thymidine for 1 hour. Cells were washed twice with cold PBS and fixed with 5% trichloroacetic acid for 10 minutes. Cells were treated with 70% ethanol, lysed with 1% SDS/10mM NaOH, Incorporated 3H -thymidine was measured in a scintillation counter.

Smad Localization

[00199]   HAEC or HUVEC cultured on glass cover slips were serum starved in EBM-2

deficient of FCS and VEGF for 6 hours. Then, the cells were treated with 10 ng/ml

BMP-2 for 0, 15, 30, 45, and 60 minutes. Cells were fixed with 3.7% formaldehyde,

permealized with 0.5% Triton X, and blocked with 1% BSA / PBS. Next, cells were

incubated overnight with a 1:50 dilution of anti-pSmad 1/5/8 antibody at 4 °C and

AlexaFluor 488 anti-goat was used as the secondary antibody (Molecular Probes, Eugene,

OR). To inhibit BMP-2, 40 ng of recombinant noggin was added to the BMP-2 treated

cells. Negative controls included primary antibody incubated with blocking peptide

(Santa Cruz) and AlexaFluor 488 without primary antibody. Smad 1/5/8 localization was

assessed by immunofluorescent microscopy.

Western Blot Analysis and Immunoprecipitation

[00200]   HAEC or HUVEC in EMB-2 with 2% FCS were treated with 10 ng/ml of

recombinant BMP-2 for 0, 10, 20, 40, 60, and 120 minutes. Western blot analysis was

performed on the cell lysate as in previous experiments. For immunoprecipitation

studies, HAEC were treated for 20 minutes with 20 ng/ml of BMP-2. Cell lysate was

incubated with anti-pSmad 1/5/8 antibody and separated by SDS-PAGE. Immunoblots

were probed with anti-phosphoserine antibody (Zymed Laboratories, San Francisco, CA).

Tube Assay

[00201]   Twenty-four well tissue culture plates were coated with 250 ul of Matrigel (BD

Biosciences). HAEC or HUVEC were plated at 5.0 x 104 in 1ml of EBM-2

supplemented with 1% FCS and all growth factors excluding VEGF. Cells were then treated with vehicle control, 500pg/ml BMP-2, or 1ng/ml BMP-2 for 18 hours at 37°C. To inhibit BMP-2, 40ng/ml of recombinant noggin was added to samples treated with BMP-2. Cells were photographed at 4X magnification and the formation of complete tubes counted in 3 fields.

BMP-2 Tumor Angiogenesis Assay

[00202]   1 X107 A549 cells were subcutaneously injected into the flanks of NCR nude mice with Affi-Blue agarose beads (Biorad, Hercules, CA) coated with BSA or recombinant human BMP-2. 25 ug of Affi-Blue agarose were incubated with 2 g of BSA or recombinant BMP-2 for 2 hours and washed with PBS. Tumors were collected 4-6 or 12-14 days later. Tumors were placed in O.C.T and frozen in liquid nitrogen.

[00203]   Blood vessels were detected by immunohistochemistry using anti-PECAM-1 antibody. Vessels were photographed and five 40X fields counted. The mice studies were approved by the Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

Matrigel Angiogenesis Assay

[00204]   2 x 106 A549, A549-AS, or A549-vector cells in 100 ul DMEM with 5% FBS were mixed with 400 ul Matrigel and injected subcutaneously into nude mice. The Matrigel was supplemented with 835 ng of BSA or 670 ng recombinant BMP-2. To inhibit BMP-2, 670 ng of recombinant BMP-2 was incubated with 835ng noggin for 2 hour at 37°C prior to mixing with the Matrigel. The Matrigel plugs were harvested after

6 days, placed in O.C.T. and frozen in liquid nitrogen. Blood vessels were detected by immunochemistry. All of the vessels within the Matrigel plugs were photographed and counted.

Immunohistochemistry

[00205]   Frozen samples were cut with a dermatome to obtain 5 micron sections. The samples were incubated with a rat anti-PECAM-1 antibody at a 1:20 dilution overnight at 4°C. The anti-rat IgG was incubated at a 1:300 dilution at room temperature for 20 minutes. Positive control included a mouse liver. Negative controls consisted of samples treated with secondary antibody alone.

Statistical Analysis

[00206]   To evaluate multiple groups a one-way ANOVA followed by Fisher LSD post hoc test was used to compare individual means. To compare 2 groups a student t-test was used. Differences with P values < 0.05 were considered statistically significant.

Cited References

1.   American Cancer Society. What Are The Key Statistics for Lung Cancer. www.cancer.org. 2003.

2.   Warburton, D., Schwarz, M., Tefft, D., Flores-Delgado, G., Anderson, K. D. & Cardoso, W. V. 2000) Mech Dev 92, 55-81.

3.   Celeste AJ, I. J., Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990) Proc Natl Acad Sci USA 87, 9843-9847.

4.   Erickson, D. M., Harris, S. E., Dean, D. D., Harris, M. A., Wozney, J. M., Boyan, B. D. & Schwartz, Z. (1997) J Orthop Res 15, 371-80.

5.   An, J., Rosen, V., Cox, K., Beauchemin, N. & Sullivan, A. K. (1996) Exp Hematol 24, 768-75.

6.   Abe, E., Yamamoto, M., Taguchi, Y., Lecka-Czemik, B., O'Brien, C. A., Economides, A. N., Stahl, N., Jilka, R. L. & Manolagas, S. C. (2000) J Bone Miner Res 15,663-73.

7.   Vainio, S., Karavanova, I., Jowett, A. & Thesleff, I. (1993) Cell 75, 45-58.

8.   Weaver, M., Yingling, J. M., Dunn, N. R., Bellusci, S. & Hogan, B. L. (1999) Development 126, 4005-15.

9.   Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. & Wang, E. A. (1988) Science 242, 1528-34.

10.   Willette, R. N., Gu, J. L., Lysko, P. G., Anderson, K. M., Minehart, H. & Yue, T. (1999) J Vasc Res 36, 120-5.

11.   Cunningham, N. S., Paralkar, V. & Reddi, A. H. (1992) Proc Natl Acad Sci U SA 89, 11740-4.

12.   Cui, Y., Jean, F., Thomas, G. & Christian, J. L. (1998) Embo J 17, 4735-43.

13.   Sugiura (1999) Biochem J 338, 443-440.

14.   Liu, F., Ventura, F., Doody, J. & Massague, J. (1995) Mol Cell Biol 15, 3479-86.

15.   Ikeda, T., Takahashi, H., Suzuki, A., Ueno, N., Yokose, S., Yamaguchi, A. & Yoshiki, S. (1996) Dev Dyvn 206, 3 18-29.

16.   Ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C. H. & Miyazono, K. (1993) <u>Oncogene</u> 8, 2879-87.

17.   Ten Dijke, P., Yamashita, H., Sampath, T. K., Reddi, A. H., Estevez, M., Riddle, D. L., Ichijo, H., Heldin, C. H. & Miyazono, K. (1994) <u>J Biol Chem</u> 269,16985-8.

18.   Sakou, T., Onishi, T., Yamamoto, T., Nagamine, Sampath, T. & Ten Dijke, P. (1999) <u>J Bone Miner Res</u> 14, 1145-52.

19.   Guo, W., et al. "Expression of bone morphogenetic proteins and receptors in sarcomas" <u>Clin. Orthop</u>. 365: 175-83 (1999).

20.   Kleef, J., "Bone Morphogenetic Protein-2 exerts diverse effects on cell growth in vitro and is expressed in human pancreatic cancer in vivo" <u>Gastroenterology</u> 116: 1202-1216 (1999).

21.   Hatakeyama, S., et al., "Expression of bone morphogenetic proteins of human neoplastic epithelial cells" <u>Biochem Mol. Biol. Int</u>. 42(3): 497 (1997).

22.   Kawamura, C., et al., "Bone morphogenetic protein-2 induces apoptosis in human myeloma cells with modulation of STAT3" <u>Blood</u> 96(6): 2005-11 (2000).

23.   Soda, H. "Antiproliferative effects of recombinant human bone morphogenetic protein-2 on human tumor colony-forming units" <u>Anticancer Drugs</u> 9(4): 327-31 (1998).

24.   Tada, A., et al., "Bone morphogenetic protein-2 suppresses the transformed phenotype and restores actin microfilaments of human lung carcinoma A549 cells" Oncol. Rep. 5(5): 137-40 (1998).

25.   Tanaka, K ., Abe, M ., and Sato, Y . Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of basic fibroblast growth factor in endothelial cells during angiogenesis, Jpn J Cancer Res. 90:647-54., 1999.

26.   Wu, L.W ., Mayo, L.D ., Dunbar, J.D ., Kessler, K.M ., Baerwald, M .R ., Jaffe, E .A ., Wang, D ., Warren, R .S ., and Donner, D .B . Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation, J BiolChem .275:5096-103., 2000.

27.   Liu, S ., Yu, D ., Xu, Z .P ., Riordan, J .F ., and Hu, G .F . Angiogenin activates Erk1/2 in human umbilical vein endothelial cells, Biochem Biophys Res Commun.287:305-10., 2001.

28. Alani, et al., "Immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1," Cell Biology, Vol. 96, Issue 17, 9637-9641, 1999.

29. Folkman, J. J. Nat'l Cancer Inst. 82:4 (1990).

30. Zetter, B. Annual Rev. Med. 49:407 (1998); Ferrara, N. Current Topics Microbiol. Immunol. 237:1 (1999).

31. Oro, A.E., et al., "Basal carcinomas in mice overexpressing sonic hedgehog" Science 276: 817-21 (1997).

32. Zimmerman, L.B., et al., Cell 86: 599-606 (1996).

33. Tucker, A.S., et al., Science 282: 1136-1138 (1998).

34. Capdevilla, J., et al., Developmental Biology 197: 205-217 (1998).

35. Brunet, L.J., et al., Science 280: 1455-1447 (1998).

36. Millet, C., et al., "The human chordin gene encodes several differentially spliced variants with distinct BMP opposing activities" Mech. Dev. 106(1-2): 85-96 (2001).

37. Nickel, J., et al. "The Crystal Structure of the BMP-2:BMPR-IA Complex and the Generation of BMP-2 Antagonists" The Journal of Bone & Joint Surgery 83-A, Supp.1, Part 1: 7-14 (2001).

38. Kirsch, T., et al. "BMP-2 antagonists emerge from alterations in the low-affinity binding epitope for receptor BMPR-II" The EMBO Journal 19(13):3314-24 (2000).

39. Ide, H., et al., "Cloning of human bone morphogenetic protein type IB receptor (BMPR-IB) and its expression in prostate cancer in comparison with other BMPRs" Oncogene 13(11): 1377-82 (1997).

40. Cameron, A., et al., "Polyarginines are potent furin inhibitors" J. Biol. Chem. 275: 36741-49 (2000).

41. Glavic, A., et al., "Xiro-1 controls mesoderm patterning by repressing BMP-4 expression in the Spemann organizer" Dev. Dyn.222(3): 368-376.

42. E. Hay, et al., "Bone Morphogenetic Protein-2 Promotes Osteoblast Apoptosis through a Smad-independent, Protein Kinase C-dependent Signaling Pathway" J. Biol. Chem., August 3, 2001; 276(31): 29028 - 29036.

43.   Chen, et al., "Suppression of Tumor Mediated Necrosis Factor Apoptosis by Nuclear Factor B Independent Bone Morphogenetic Bone/Smad Signalling," J. Biol. Chem., Vol. 276, Issue 42, 39259-39263, 2001.

44.   Izumi et al., "Bone Morphogenetic Protein-2 Inhibits Serum Depravation Induced Apoptosis of Neonatal Cardiac Myocytes through Activation of the Smad-1 Pathway," J. Biol. Chem., Vol. 276, Issue 33, 31133-31141, 2001.

45.   Ogata, et al., "Bone Morphogenetic Protein 2 Transiently Enhances Expression of a Gene, Id (Inhibitor of Differentiation), Encoding a Helix-Loop-Helix Molecule in Osteoblast-Like Cells," Proc. Natl. Acad. Sci. USA. 1993; 90 (19): 9219–9222.

46.   Locklin et al., "Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology," J Bone Miner Res. 2001 Dec;16(12):2192-204.

47.   Hollnagel, et al., "*Id* Genes Are Direct Targets of Bone Morphogenetic Protein Induction in Embryonic Stem Cells," J Biol Chem, Vol. 274, Issue 28, 19838-19845, 1999.

48.   Zebedee, et al., "Id proteins in cell cycle control and cellular senescence," Oncogene 20 (2001) 8317–8325.

49.   Schindl, et al., "Level of Id-1 protein expression correlates with poor differentiation, enhanced malignant potential, and more aggressive clinical behavior of epithelial ovarian tumors," Clin Cancer Res 9: 779-785 (2003).

Other References

Thoracic Surgery (Churchill Livingstone, New York, Edinburgh, London, Melbourne, Tokyo) (1995).

SEQUENCE LISTING

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<302>   Identification of mammalian noggin and its expression in the

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<302>   Heterozygous mutations in the gene encoding noggin affect human
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atg gag cgc tgc ccc agc cta ggg gtc acc ctc tac gcc ctg gtg gtg   48
Met Glu Arg Cys Pro Ser Leu Gly Val Thr Leu Tyr Ala Leu Val Val
1   5   10   15

gtc ctg ggg ctg cgg gcg aca ccg gcc ggc ggc cag cac tat ctc cac   96
Val Leu Gly Leu Arg Ala Thr Pro Ala Gly Gly Gln His Tyr Leu His
            20   25   30

atc cgc ccg gca ccc agc gac aac ctg ccc ctg gtg gac ctc atc gaa   144
Ile Arg Pro Ala Pro Ser Asp Asn Leu Pro Leu Val Asp Leu Ile Glu
        35   40   45

cac cca gac cct atc ttt gac ccc aag gaa aag gat ctg aac gag acg   192
His Pro Asp Pro Ile Phe Asp Pro Lys Glu Lys Asp Leu Asn Glu Thr
    50   55   60

ctg ctg cgc tcg ctg ctc ggg ggc cac tac gac cca ggc ttc atg gcc   240
Leu Leu Arg Ser Leu Leu Gly Gly His Tyr Asp Pro Gly Phe Met Ala
65   70   75   80

acc tcg ccc ccc gag gac cgg ccc ggc ggg ggc ggg ggt gca gct ggg   288
Thr Ser Pro Pro Glu Asp Arg Pro Gly Gly Gly Gly Gly Ala Ala Gly
                85   90   95

ggc gcg gag gac ctg gcg gag ctg gac cag ctg ctg cgg cag cgg ccg   336
Gly Ala Glu Asp Leu Ala Glu Leu Asp Gln Leu Leu Arg Gln Arg Pro
            100   105   110

tcg ggg gcc atg ccg agc gag atc aaa ggg cta gag ttc tcc gag ggc   384
Ser Gly Ala Met Pro Ser Glu Ile Lys Gly Leu Glu Phe Ser Glu Gly
            115   120   125

ttg gcc cag ggc aag aag cag cgc cta agc aag aag ctg cgg agg aag   432
Leu Ala Gln Gly Lys Lys Gln Arg Leu Ser Lys Lys Leu Arg Arg Lys
    130   135   140

```
tta cag atg tgg ctg tgg tcg cag aca ttc tgc ccc gtg ctg tac gcg     480
Leu Gln Met Trp Leu Trp Ser Gln Thr Phe Cys Pro Val Leu Tyr Ala
145             150             155             160

tgg aac gac ctg ggc agc cgc ttt tgg ccg cgc tac gtg aag gtg ggc     528
Trp Asn Asp Leu Gly Ser Arg Phe Trp Pro Arg Tyr Val Lys Val Gly
                165             170             175

agc tgc ttc agt aag cgc tcg tgc tcc gtg ccc gag ggc atg gtg tgc     576
Ser Cys Phe Ser Lys Arg Ser Cys Ser Val Pro Glu Gly Met Val Cys
            180             185             190

aag ccg tcc aag tcc gtg cac ctc acg gtg ctg cgg tgg cgc tgt cag     624
Lys Pro Ser Lys Ser Val His Leu Thr Val Leu Arg Trp Arg Cys Gln
            195             200             205

cgg cgc ggg ggc cag cgc tgc ggc tgg att ccc atc cag tac ccc atc     672
Arg Arg Gly Gly Gln Arg Cys Gly Trp Ile Pro Ile Gln Tyr Pro Ile
210             215             220

att tcc gag tgc aag tgc tcg tgc tag                                 699
Ile Ser Glu Cys Lys Cys Ser Cys
225             230
```

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<210>  4
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<212>  PRT
<213>  Homo sapiens

<400>  4
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Met Glu Arg Cys Pro Ser Leu Gly Val Thr Leu Tyr Ala Leu Val Val
1               5               10              15


Val Leu Gly Leu Arg Ala Thr Pro Ala Gly Gly Gln His Tyr Leu His
            20              25              30


Ile Arg Pro Ala Pro Ser Asp Asn Leu Pro Leu Val Asp Leu Ile Glu
            35              40              45


His Pro Asp Pro Ile Phe Asp Pro Lys Glu Lys Asp Leu Asn Glu Thr
        50              55              60


Leu Leu Arg Ser Leu Leu Gly Gly His Tyr Asp Pro Gly Phe Met Ala
65              70              75              80


Thr Ser Pro Pro Glu Asp Arg Pro Gly Gly Gly Gly Gly Ala Ala Gly
                85              90              95


Gly Ala Glu Asp Leu Ala Glu Leu Asp Gln Leu Leu Arg Gln Arg Pro
                100             105             110
```

```
Ser Gly Ala Met Pro Ser Glu Ile Lys Gly Leu Glu Phe Ser Glu Gly
        115                 120                 125

Leu Ala Gln Gly Lys Lys Gln Arg Leu Ser Lys Lys Leu Arg Arg Lys
    130                 135                 140

Leu Gln Met Trp Leu Trp Ser Gln Thr Phe Cys Pro Val Leu Tyr Ala
145                 150                 155                 160

Trp Asn Asp Leu Gly Ser Arg Phe Trp Pro Arg Tyr Val Lys Val Gly
                165                 170                 175

Ser Cys Phe Ser Lys Arg Ser Cys Ser Val Pro Glu Gly Met Val Cys
            180                 185                 190

Lys Pro Ser Lys Ser Val His Leu Thr Val Leu Arg Trp Arg Cys Gln
        195                 200                 205

Arg Arg Gly Gly Gln Arg Cys Gly Trp Ile Pro Ile Gln Tyr Pro Ile
    210                 215                 220

Ile Ser Glu Cys Lys Cys Ser Cys
225                 230
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<210>   5
<211>   699
<212>   DNA
<213>   Mus musculus


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<221>   gene
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<223>   nog

<220>
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atg gag cgc tgc ccc agc ctg ggg gtc acc ctc tac gcc ctg gtg gtg      48
Met Glu Arg Cys Pro Ser Leu Gly Val Thr Leu Tyr Ala Leu Val Val
1               5                   10                  15

gtc ctg ggg ctg cgg gca gca cca gcc ggc ggc cag cac tat cta cac      96
Val Leu Gly Leu Arg Ala Ala Pro Ala Gly Gly Gln His Tyr Leu His
                20                  25                  30

atc cgc cca gca ccc agc gac aac ctg ccc ttg gtg gac ctc atc gaa     144
Ile Arg Pro Ala Pro Ser Asp Asn Leu Pro Leu Val Asp Leu Ile Glu
        35                  40                  45
```

```
cat cca gac cct atc ttt gac cct aag gag aag gat ctg aac gag acg      192
His Pro Asp Pro Ile Phe Asp Pro Lys Glu Lys Asp Leu Asn Glu Thr
    50              55              60

ctg ctg cgc tcg ctg ctc ggg ggc cac tac gac ccg ggc ttt atg gcc      240
Leu Leu Arg Ser Leu Leu Gly Gly His Tyr Asp Pro Gly Phe Met Ala
65              70              75              80

act tcg ccc cca gag gac cga ccc gga ggg ggc ggg gga ccg gct gga      288
Thr Ser Pro Pro Glu Asp Arg Pro Gly Gly Gly Gly Gly Pro Ala Gly
                85              90              95

ggt gcc gag gac ctg gcg gag ctg gac cag ctg ctg cgg cag cgg ccg      336
Gly Ala Glu Asp Leu Ala Glu Leu Asp Gln Leu Leu Arg Gln Arg Pro
                100             105             110

tcg ggg gcc atg ccg agc gag atc aaa ggg ctg gag ttc tcc gag ggc      384
Ser Gly Ala Met Pro Ser Glu Ile Lys Gly Leu Glu Phe Ser Glu Gly
            115             120             125

ttg gcc caa ggc aag aaa cag cgc ctg agc aag aag ctg agg agg aag      432
Leu Ala Gln Gly Lys Lys Gln Arg Leu Ser Lys Lys Leu Arg Arg Lys
        130             135             140

tta cag atg tgg ctg tgg tca cag acc ttc tgc ccg gtg ctg tac gcg      480
Leu Gln Met Trp Leu Trp Ser Gln Thr Phe Cys Pro Val Leu Tyr Ala
145             150             155             160

tgg aat gac cta ggc agc cgc ttt tgg cca cgc tac gtg aag gtg ggc      528
Trp Asn Asp Leu Gly Ser Arg Phe Trp Pro Arg Tyr Val Lys Val Gly
                165             170             175

agc tgc ttc agc aag cgc tcc tgc tct gtg ccc gag ggc atg gtg tgt      576
Ser Cys Phe Ser Lys Arg Ser Cys Ser Val Pro Glu Gly Met Val Cys
            180             185             190

aag cca tcc aag tct gtg cac ctc acg gtg ctg cgg tgg cgc tgt cag      624
Lys Pro Ser Lys Ser Val His Leu Thr Val Leu Arg Trp Arg Cys Gln
        195             200             205

cgg cgc ggg ggt cag cgc tgc ggc tgg att ccc atc cag tac ccc atc      672
Arg Arg Gly Gly Gln Arg Cys Gly Trp Ile Pro Ile Gln Tyr Pro Ile
    210             215             220

att tcc gag tgt aag tgt tcc tgc tag                                   699
Ile Ser Glu Cys Lys Cys Ser Cys
225             230
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<210>   6
<211>   232
<212>   PRT
<213>   Mus musculus

<400>   6

Met Glu Arg Cys Pro Ser Leu Gly Val Thr Leu Tyr Ala Leu Val Val
1               5               10              15


Val Leu Gly Leu Arg Ala Ala Pro Ala Gly Gly Gln His Tyr Leu His
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              20                      25                      30

Ile Arg Pro Ala Pro Ser Asp Asn Leu Pro Leu Val Asp Leu Ile Glu
        35              40              45

His Pro Asp Pro Ile Phe Asp Pro Lys Glu Lys Asp Leu Asn Glu Thr
        50              55              60

Leu Leu Arg Ser Leu Leu Gly Gly His Tyr Asp Pro Gly Phe Met Ala
65              70              75              80

Thr Ser Pro Pro Glu Asp Arg Pro Gly Gly Gly Gly Pro Ala Gly
            85              90              95

Gly Ala Glu Asp Leu Ala Glu Leu Asp Gln Leu Leu Arg Gln Arg Pro
            100             105             110

Ser Gly Ala Met Pro Ser Glu Ile Lys Gly Leu Glu Phe Ser Glu Gly
        115             120             125

Leu Ala Gln Gly Lys Lys Gln Arg Leu Ser Lys Lys Leu Arg Arg Lys
        130             135             140

Leu Gln Met Trp Leu Trp Ser Gln Thr Phe Cys Pro Val Leu Tyr Ala
145             150             155             160

Trp Asn Asp Leu Gly Ser Arg Phe Trp Pro Arg Tyr Val Lys Val Gly
            165             170             175

Ser Cys Phe Ser Lys Arg Ser Cys Ser Val Pro Glu Gly Met Val Cys
        180             185             190

Lys Pro Ser Lys Ser Val His Leu Thr Val Leu Arg Trp Arg Cys Gln
        195             200             205

Arg Arg Gly Gly Gln Arg Cys Gly Trp Ile Pro Ile Gln Tyr Pro Ile
    210             215             220

Ile Ser Glu Cys Lys Cys Ser Cys
225             230
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<210>  7
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<212>  DNA
<213>  Homo sapiens

<300>
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<301>  Millet, C., Lemaire, P., Orsetti, B., Guglielmi, P., and
       Francois, V.
<302>  The human chordin gene encodes several differentially expressed
       spliced variants with distinct BMP opposing activities
<303>  Mech. Dev.
<304>  106
<305>  1
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<308>  AF209928
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<313>  (1)..(3547)

<300>
<301>  Millet, C., and Francois, V.
<302>  Direct Submission
<303>  Institut de Genetique Humaine
<304>  1
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<307>  1999-11-30
<308>  AF209928
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cggcgccgac tctgcggccg cccgacgagc ccctcgcggc actgccccgg ccccggcccc    120

ggccccggcc ccctcccgcc gcaccgcccc cggcccggcc ctccgccctc cgcactcccg    180

cctccctccc tccgcccgct cccgcgccct cctccctccc tcctccccag ctgtcccgtt    240

cgcgtc atg ccg agc ctc ccg gcc ccg ccg gcc ccg ctg ctg ctc ctc      288
        Met Pro Ser Leu Pro Ala Pro Pro Ala Pro Leu Leu Leu Leu
         1               5                     10

ggg ctg ctg ctg ctc ggc tcc cgg ccg gcc cgc ggc gcc ggc ccc gag    336
Gly Leu Leu Leu Leu Gly Ser Arg Pro Ala Arg Gly Ala Gly Pro Glu
15                  20                  25                  30

ccc ccc gtg ctg ccc atc cgt tct gag aag gag ccg ctg ccc gtt cgg    384
Pro Pro Val Leu Pro Ile Arg Ser Glu Lys Glu Pro Leu Pro Val Arg
                35                  40                  45
```

```
gga gcg gca ggc tgc acc ttc ggc ggg aag gtc tat gcc ttg gac gag      432
Gly Ala Ala Gly Cys Thr Phe Gly Gly Lys Val Tyr Ala Leu Asp Glu
            50              55                  60

acg tgg cac ccg gac cta ggg gag cca ttc ggg gtg atg cgc tgc gtg      480
Thr Trp His Pro Asp Leu Gly Glu Pro Phe Gly Val Met Arg Cys Val
            65              70                  75

ctg tgc gcc tgc gag gcg cct cag tgg ggt cgc cgt acc agg ggc cct      528
Leu Cys Ala Cys Glu Ala Pro Gln Trp Gly Arg Arg Thr Arg Gly Pro
    80              85                  90

ggc agg gtc agc tgc aag aac atc aaa cca gag tgc cca acc ccg gcc      576
Gly Arg Val Ser Cys Lys Asn Ile Lys Pro Glu Cys Pro Thr Pro Ala
95              100                 105                 110

tgt ggg cag ccg cgc cag ctg ccg gga cac tgc tgc cag acc tgc ccc      624
Cys Gly Gln Pro Arg Gln Leu Pro Gly His Cys Cys Gln Thr Cys Pro
                115                 120                 125

cag gag cgc agc agt tcg gag cgg cag ccg agc ggc ctg tcc ttc gag      672
Gln Glu Arg Ser Ser Ser Glu Arg Gln Pro Ser Gly Leu Ser Phe Glu
            130                 135                 140

tat ccg cgg gac ccg gag cat cgc agt tat agc gac cgc ggg gag cca      720
Tyr Pro Arg Asp Pro Glu His Arg Ser Tyr Ser Asp Arg Gly Glu Pro
            145                 150                 155

ggc gct gag gag cgg gcc cgt ggt gac ggc cac acg gac ttc gtg gcg      768
Gly Ala Glu Glu Arg Ala Arg Gly Asp Gly His Thr Asp Phe Val Ala
    160                 165                 170

ctg ctg aca ggg ccg agg tcg cag gcg gtg gca cga gcc cga gtc tcg      816
Leu Leu Thr Gly Pro Arg Ser Gln Ala Val Ala Arg Ala Arg Val Ser
175                 180                 185                 190

ctg ctg cgc tct agc ctc cgc ttc tct atc tcc tac agg cgg ctg gac      864
Leu Leu Arg Ser Ser Leu Arg Phe Ser Ile Ser Tyr Arg Arg Leu Asp
                195                 200                 205

cgc cct acc agg atc cgc ttc tca gac tcc aat ggc agt gtc ctg ttt      912
Arg Pro Thr Arg Ile Arg Phe Ser Asp Ser Asn Gly Ser Val Leu Phe
            210                 215                 220

gag cac cct gca gcc ccc acc caa gat ggc ctg gtc tgt ggg gtg tgg      960
Glu His Pro Ala Ala Pro Thr Gln Asp Gly Leu Val Cys Gly Val Trp
            225                 230                 235

cgg gca gtg cct cgg ttg tct ctg cgg ctc ctt agg gca gaa cag ctg     1008
Arg Ala Val Pro Arg Leu Ser Leu Arg Leu Leu Arg Ala Glu Gln Leu
    240                 245                 250

cat gtg gca ctt gtg aca ctc act cac cct tca ggg gag gtc tgg ggg     1056
His Val Ala Leu Val Thr Leu Thr His Pro Ser Gly Glu Val Trp Gly
255                 260                 265                 270

cct ctc atc cgg cac cgg gcc ctg gct gca gag acc ttc agt gcc atc     1104
Pro Leu Ile Arg His Arg Ala Leu Ala Ala Glu Thr Phe Ser Ala Ile
                275                 280                 285
```

```
ctg act cta gaa ggc ccc cca cag cag ggc gta ggg ggc atc acc ctg         1152
Leu Thr Leu Glu Gly Pro Pro Gln Gln Gly Val Gly Gly Ile Thr Leu
            290                 295                 300

ctc act ctc agt gac aca gag gac tcc ttg cat ttt ttg ctg ctc ttc         1200
Leu Thr Leu Ser Asp Thr Glu Asp Ser Leu His Phe Leu Leu Leu Phe
            305                 310                 315

cga ggg ctg ctg gaa ccc agg agt ggg gga cta acc cag gtt ccc ttg         1248
Arg Gly Leu Leu Glu Pro Arg Ser Gly Gly Leu Thr Gln Val Pro Leu
            320                 325                 330

agg ctc cag att cta cac cag ggg cag cta ctg cga gaa ctt cag gcc         1296
Arg Leu Gln Ile Leu His Gln Gly Gln Leu Leu Arg Glu Leu Gln Ala
335                 340                 345                 350

aat gtc tca gcc cag gaa cca ggc ttt gct gag gtg ctg ccc aac ctg         1344
Asn Val Ser Ala Gln Glu Pro Gly Phe Ala Glu Val Leu Pro Asn Leu
                355                 360                 365

aca gtc cag gag atg gac tgg ctg gtg ctg ggg gag ctg cag atg gcc         1392
Thr Val Gln Glu Met Asp Trp Leu Val Leu Gly Glu Leu Gln Met Ala
            370                 375                 380

ctg gag tgg gca ggc agg cca ggg ctg cgc atc agt gga cac att gct         1440
Leu Glu Trp Ala Gly Arg Pro Gly Leu Arg Ile Ser Gly His Ile Ala
            385                 390                 395

gcc agg aag agc tgc gac gtc ctg caa agt gtc ctt tgt ggg gct gat         1488
Ala Arg Lys Ser Cys Asp Val Leu Gln Ser Val Leu Cys Gly Ala Asp
        400                 405                 410

gcc ctg atc cca gtc cag acg ggt gct gcc ggc tca gcc agc ctc acg         1536
Ala Leu Ile Pro Val Gln Thr Gly Ala Ala Gly Ser Ala Ser Leu Thr
415                 420                 425                 430

ctg cta gga aat ggc tcc ctg atc tat cag gtg caa gtg gta ggg aca         1584
Leu Leu Gly Asn Gly Ser Leu Ile Tyr Gln Val Gln Val Val Gly Thr
                435                 440                 445

agc agt gag gtg gtg gcc atg aca ctg gag acc aag cct cag cgg agg         1632
Ser Ser Glu Val Val Ala Met Thr Leu Glu Thr Lys Pro Gln Arg Arg
            450                 455                 460

gat cag cgc act gtc ctg tgc cac atg gct gga ctc cag cca gga gga         1680
Asp Gln Arg Thr Val Leu Cys His Met Ala Gly Leu Gln Pro Gly Gly
            465                 470                 475

cac acg gcc gtg ggt atc tgc cct ggg ctg ggt gcc cga ggg gct cat         1728
His Thr Ala Val Gly Ile Cys Pro Gly Leu Gly Ala Arg Gly Ala His
            480                 485                 490

atg ctg ctg cag aat gag ctc ttc ctg aat gtg ggc acc aag gac ttc         1776
Met Leu Leu Gln Asn Glu Leu Phe Leu Asn Val Gly Thr Lys Asp Phe
495                 500                 505                 510

cca gac gga gag ctt cgg ggg cac gtg gct gcc ctg ccc tac tgt ggg         1824
Pro Asp Gly Glu Leu Arg Gly His Val Ala Ala Leu Pro Tyr Cys Gly
            515                 520                 525

cat agc gcc cgc cat gac acg ctg ccc gtg ccc cta gca gga gcc ctg         1872
```

```
His Ser Ala Arg His Asp Thr Leu Pro Val Pro Leu Ala Gly Ala Leu
            530         535             540

gtg cta ccc cct gtg aag agc caa gca gca ggg cac gcc tgg ctt tcc      1920
Val Leu Pro Pro Val Lys Ser Gln Ala Ala Gly His Ala Trp Leu Ser
            545         550             555

ttg gat acc cac tgt cac ctg cac tat gaa gtg ctg ctg gct ggg ctt      1968
Leu Asp Thr His Cys His Leu His Tyr Glu Val Leu Leu Ala Gly Leu
            560         565             570

ggt ggc tca gaa caa ggc act gtc act gcc cac ctc ctt ggg cct cct      2016
Gly Gly Ser Glu Gln Gly Thr Val Thr Ala His Leu Leu Gly Pro Pro
575             580             585             590

gga acg cca ggg cct cgg cgg ctg ctg aag gga ttc tat ggc tca gag      2064
Gly Thr Pro Gly Pro Arg Arg Leu Leu Lys Gly Phe Tyr Gly Ser Glu
                595             600             605

gcc cag ggt gtg gtg aag gac ctg gag ccg gaa ctg ctg cgg cac ctg      2112
Ala Gln Gly Val Val Lys Asp Leu Glu Pro Glu Leu Leu Arg His Leu
            610             615             620

gca aaa ggc atg gcc tcc ctg ctg atc acc acc aag ggt agc ccc aga      2160
Ala Lys Gly Met Ala Ser Leu Leu Ile Thr Thr Lys Gly Ser Pro Arg
            625             630             635

ggg gag ctc cga ggg cag gtg cac ata gcc aac caa tgt gag gtt ggc      2208
Gly Glu Leu Arg Gly Gln Val His Ile Ala Asn Gln Cys Glu Val Gly
            640             645             650

gga ctg cgc ctg gag gcg gcc ggg gcc gag ggg gtg cgg gcg ctg ggg      2256
Gly Leu Arg Leu Glu Ala Ala Gly Ala Glu Gly Val Arg Ala Leu Gly
655             660             665             670

gct ccg gat aca gcc tct gct gcg ccg cct gtg gtg cct ggt ctc ccg      2304
Ala Pro Asp Thr Ala Ser Ala Ala Pro Pro Val Val Pro Gly Leu Pro
            675             680             685

gcc cta gcg ccc gcc aaa cct ggt ggt cct ggg cgg ccc cga gac ccc      2352
Ala Leu Ala Pro Ala Lys Pro Gly Gly Pro Gly Arg Pro Arg Asp Pro
            690             695             700

aac aca tgc ttc ttc gag ggg cag cag cgc ccc cac ggg gct cgc tgg      2400
Asn Thr Cys Phe Phe Glu Gly Gln Gln Arg Pro His Gly Ala Arg Trp
            705             710             715

gcg ccc aac tac gac ccg ctc tgc tca ctc tgc acc tgc cag aga cga      2448
Ala Pro Asn Tyr Asp Pro Leu Cys Ser Leu Cys Thr Cys Gln Arg Arg
            720             725             730

acg gtg atc tgt gac ccg gtg gtg tgc cca ccg ccc agc tgc cca cac      2496
Thr Val Ile Cys Asp Pro Val Val Cys Pro Pro Pro Ser Cys Pro His
735             740             745             750

ccg gtg cag gct ccc gac cag tgc tgc cct gtt tgc cct gag aaa caa      2544
Pro Val Gln Ala Pro Asp Gln Cys Cys Pro Val Cys Pro Glu Lys Gln
            755             760             765

gat gtc aga gac ttg cca ggg ctg cca agg agc cgg gac cca gga gag      2592
Asp Val Arg Asp Leu Pro Gly Leu Pro Arg Ser Arg Asp Pro Gly Glu
```

```
                770                      775                      780

ggc tgc tat ttt gat ggt gac cgg agc tgg cgg gca gcg ggt acg cgg      2640
Gly Cys Tyr Phe Asp Gly Asp Arg Ser Trp Arg Ala Ala Gly Thr Arg
            785                      790                      795

tgg cac ccc gtt gtg ccc ccc ttt ggc tta att aag tgt gct gtc tgc      2688
Trp His Pro Val Val Pro Pro Phe Gly Leu Ile Lys Cys Ala Val Cys
        800                      805                      810

acc tgc aag ggg ggc act gga gag gtg cac tgt gag aag gtg cag tgt      2736
Thr Cys Lys Gly Gly Thr Gly Glu Val His Cys Glu Lys Val Gln Cys
815                      820                      825                      830

ccc cgg ctg gcc tgt gcc cag cct gtg cgt gtc aac ccc acc gac tgc      2784
Pro Arg Leu Ala Cys Ala Gln Pro Val Arg Val Asn Pro Thr Asp Cys
                    835                      840                      845

tgc aaa cag tgt cca gtg ggg tcg ggg gcc cac ccc cag ctg ggg gac      2832
Cys Lys Gln Cys Pro Val Gly Ser Gly Ala His Pro Gln Leu Gly Asp
                850                      855                      860

ccc atg cag gct gat ggg ccc cgg ggc tgc cgt ttt gct ggg cag tgg      2880
Pro Met Gln Ala Asp Gly Pro Arg Gly Cys Arg Phe Ala Gly Gln Trp
            865                      870                      875

ttc cca gag agt cag agc tgg cac ccc tca gtg ccc cct ttt gga gag      2928
Phe Pro Glu Ser Gln Ser Trp His Pro Ser Val Pro Pro Phe Gly Glu
        880                      885                      890

atg agc tgt atc acc tgc aga tgt ggg gca ggg gtg cct cac tgt gag      2976
Met Ser Cys Ile Thr Cys Arg Cys Gly Ala Gly Val Pro His Cys Glu
895                      900                      905                      910

cgg gat gac tgt tca ctg cca ctg tcc tgt ggc tcg ggg aag gag agt      3024
Arg Asp Asp Cys Ser Leu Pro Leu Ser Cys Gly Ser Gly Lys Glu Ser
                    915                      920                      925

cga tgc tgt tcc cgc tgc acg gcc cac cgg cgg cca gcc cca gag acc      3072
Arg Cys Cys Ser Arg Cys Thr Ala His Arg Arg Pro Ala Pro Glu Thr
                930                      935                      940

aga act gat cca gag ctg gag aaa gaa gcc gaa ggc tct tag            3114
Arg Thr Asp Pro Glu Leu Glu Lys Glu Ala Glu Gly Ser
            945                      950                      955

ggagcagcca gagggccaag tgaccaagag gatggggcct gagctgggga aggggtggca      3174

tcgaggacct tcttgcattc tcctgtggga agcccagtgc ctttgctcct ctgtcctgcc      3234

tctactccca cccccactac ctctgggaac cacagctcca caaggggggag aggcagctgg      3294

gccagaccga ggtcacagcc actccaagtc ctgccctgcc accctcggcc tctgtcctgg      3354

aagccccacc cctttcctcc tgtacataat gtcactggct tgttgggatt tttaatttat      3414

cttcactcag caccaagggc ccccgacact ccactcctgc tgcccctgag ctgagcagag      3474

tcattattgg agagttttgt atttattaaa acatttcttt ttcagtcaaa aaaaaaaaa       3534

aaaaaaaaaa aaa                                                          3547
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<210>   8
<211>   955
<212>   PRT
<213>   Homo sapiens

<400>   8

Met Pro Ser Leu Pro Ala Pro Pro Ala Pro Leu Leu Leu Leu Gly Leu
1               5                   10                  15

Leu Leu Leu Gly Ser Arg Pro Ala Arg Gly Ala Gly Pro Glu Pro Pro
            20                  25                  30

Val Leu Pro Ile Arg Ser Glu Lys Glu Pro Leu Pro Val Arg Gly Ala
        35                  40                  45

Ala Gly Cys Thr Phe Gly Gly Lys Val Tyr Ala Leu Asp Glu Thr Trp
    50                  55                  60

His Pro Asp Leu Gly Glu Pro Phe Gly Val Met Arg Cys Val Leu Cys
65                  70                  75                  80

Ala Cys Glu Ala Pro Gln Trp Gly Arg Arg Thr Arg Gly Pro Gly Arg
                85                  90                  95

Val Ser Cys Lys Asn Ile Lys Pro Glu Cys Pro Thr Pro Ala Cys Gly
            100                 105                 110

Gln Pro Arg Gln Leu Pro Gly His Cys Cys Gln Thr Cys Pro Gln Glu
            115                 120                 125

Arg Ser Ser Ser Glu Arg Gln Pro Ser Gly Leu Ser Phe Glu Tyr Pro
    130                 135                 140

Arg Asp Pro Glu His Arg Ser Tyr Ser Asp Arg Gly Glu Pro Gly Ala
145                 150                 155                 160

Glu Glu Arg Ala Arg Gly Asp Gly His Thr Asp Phe Val Ala Leu Leu
                165                 170                 175

Thr Gly Pro Arg Ser Gln Ala Val Ala Arg Ala Arg Val Ser Leu Leu
                180                 185                 190

Arg Ser Ser Leu Arg Phe Ser Ile Ser Tyr Arg Arg Leu Asp Arg Pro
    195                 200                 205
```

```
Thr Arg Ile Arg Phe Ser Asp Ser Asn Gly Ser Val Leu Phe Glu His
    210             215             220

Pro Ala Ala Pro Thr Gln Asp Gly Leu Val Cys Gly Val Trp Arg Ala
225             230             235             240

Val Pro Arg Leu Ser Leu Arg Leu Leu Arg Ala Glu Gln Leu His Val
            245             250             255

Ala Leu Val Thr Leu Thr His Pro Ser Gly Glu Val Trp Gly Pro Leu
            260             265             270

Ile Arg His Arg Ala Leu Ala Ala Glu Thr Phe Ser Ala Ile Leu Thr
        275             280             285

Leu Glu Gly Pro Pro Gln Gln Gly Val Gly Gly Ile Thr Leu Leu Thr
    290             295             300

Leu Ser Asp Thr Glu Asp Ser Leu His Phe Leu Leu Leu Phe Arg Gly
305             310             315             320

Leu Leu Glu Pro Arg Ser Gly Gly Leu Thr Gln Val Pro Leu Arg Leu
            325             330             335

Gln Ile Leu His Gln Gly Gln Leu Leu Arg Glu Leu Gln Ala Asn Val
        340             345             350

Ser Ala Gln Glu Pro Gly Phe Ala Glu Val Leu Pro Asn Leu Thr Val
        355             360             365

Gln Glu Met Asp Trp Leu Val Leu Gly Glu Leu Gln Met Ala Leu Glu
    370             375             380

Trp Ala Gly Arg Pro Gly Leu Arg Ile Ser Gly His Ile Ala Ala Arg
385             390             395             400

Lys Ser Cys Asp Val Leu Gln Ser Val Leu Cys Gly Ala Asp Ala Leu
            405             410             415

Ile Pro Val Gln Thr Gly Ala Ala Gly Ser Ala Ser Leu Thr Leu Leu
        420             425             430

Gly Asn Gly Ser Leu Ile Tyr Gln Val Gln Val Val Gly Thr Ser Ser
        435             440             445

Glu Val Val Ala Met Thr Leu Glu Thr Lys Pro Gln Arg Arg Asp Gln
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        450                 455                 460


Arg Thr Val Leu Cys His Met Ala Gly Leu Gln Pro Gly Gly His Thr
465             470             475             480


Ala Val Gly Ile Cys Pro Gly Leu Gly Ala Arg Gly Ala His Met Leu
                485             490             495


Leu Gln Asn Glu Leu Phe Leu Asn Val Gly Thr Lys Asp Phe Pro Asp
            500             505             510


Gly Glu Leu Arg Gly His Val Ala Ala Leu Pro Tyr Cys Gly His Ser
            515             520             525


Ala Arg His Asp Thr Leu Pro Val Pro Leu Ala Gly Ala Leu Val Leu
        530             535             540


Pro Pro Val Lys Ser Gln Ala Ala Gly His Ala Trp Leu Ser Leu Asp
545             550             555             560


Thr His Cys His Leu His Tyr Glu Val Leu Leu Ala Gly Leu Gly Gly
            565             570             575


Ser Glu Gln Gly Thr Val Thr Ala His Leu Leu Gly Pro Pro Gly Thr
            580             585             590


Pro Gly Pro Arg Arg Leu Leu Lys Gly Phe Tyr Gly Ser Glu Ala Gln
            595             600             605


Gly Val Val Lys Asp Leu Glu Pro Glu Leu Leu Arg His Leu Ala Lys
        610             615             620


Gly Met Ala Ser Leu Leu Ile Thr Thr Lys Gly Ser Pro Arg Gly Glu
625             630             635             640


Leu Arg Gly Gln Val His Ile Ala Asn Gln Cys Glu Val Gly Gly Leu
            645             650             655


Arg Leu Glu Ala Ala Gly Ala Glu Gly Val Arg Ala Leu Gly Ala Pro
            660             665             670


Asp Thr Ala Ser Ala Ala Pro Pro Val Val Pro Gly Leu Pro Ala Leu
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Ala Pro Ala Lys Pro Gly Gly Pro Gly Arg Pro Arg Asp Pro Asn Thr
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Cys Phe Phe Glu Gly Gln Gln Arg Pro His Gly Ala Arg Trp Ala Pro
705             710             715                     720


Asn Tyr Asp Pro Leu Cys Ser Leu Cys Thr Cys Gln Arg Arg Thr Val
                725             730                 735


Ile Cys Asp Pro Val Val Cys Pro Pro Pro Ser Cys Pro His Pro Val
            740             745                 750


Gln Ala Pro Asp Gln Cys Cys Pro Val Cys Pro Glu Lys Gln Asp Val
            755             760             765


Arg Asp Leu Pro Gly Leu Pro Arg Ser Arg Asp Pro Gly Glu Gly Cys
    770             775             780


Tyr Phe Asp Gly Asp Arg Ser Trp Arg Ala Ala Gly Thr Arg Trp His
785             790             795                     800


Pro Val Val Pro Pro Phe Gly Leu Ile Lys Cys Ala Val Cys Thr Cys
            805             810                 815


Lys Gly Gly Thr Gly Glu Val His Cys Glu Lys Val Gln Cys Pro Arg
            820             825                 830


Leu Ala Cys Ala Gln Pro Val Arg Val Asn Pro Thr Asp Cys Cys Lys
        835             840             845


Gln Cys Pro Val Gly Ser Gly Ala His Pro Gln Leu Gly Asp Pro Met
    850             855             860


Gln Ala Asp Gly Pro Arg Gly Cys Arg Phe Ala Gly Gln Trp Phe Pro
865             870             875                     880


Glu Ser Gln Ser Trp His Pro Ser Val Pro Pro Phe Gly Glu Met Ser
            885             890                 895


Cys Ile Thr Cys Arg Cys Gly Ala Gly Val Pro His Cys Glu Arg Asp
            900             905                 910


Asp Cys Ser Leu Pro Leu Ser Cys Gly Ser Gly Lys Glu Ser Arg Cys
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Cys Ser Arg Cys Thr Ala His Arg Arg Pro Ala Pro Glu Thr Arg Thr
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Asp Pro Glu Leu Glu Lys Glu Ala Glu Gly Ser
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<301> Topol, L.Z., Modi, W.S., Koochekpour, S., and Blair, D.G.
<302> DRM/Gremlin (CKTSF1B1) maps to human chromosome 15 adn is highly
      expressed in adult and fetal brain
<303> Cytogenet. Cell Genet.
<304> 89
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<300>
<301> Topol, L.Z., Marx, M., Calothy, G., and Blair, D.G.
<302> Direct Submission
<303> Oncogene Mechanisms Section, Basic Research Laboratory, NIH/NCI
<304> 1
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actgacagt atg agc cgc aca gcc tac acg gtg gga gcc ctg ctt ctc ctc   171
           Met Ser Arg Thr Ala Tyr Thr Val Gly Ala Leu Leu Leu Leu
            1   5   10

ttg ggg acc ctg ctg ccg gct gct gaa ggg aaa aag aaa ggg tcc caa   219

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Leu Gly Thr Leu Leu Pro Ala Ala Glu Gly Lys Lys Lys Gly Ser Gln
15                  20              25              30

ggt gcc atc ccc ccg cca gac aag gcc cag cac aat gac tca gag cag    267
Gly Ala Ile Pro Pro Pro Asp Lys Ala Gln His Asn Asp Ser Glu Gln
              35              40              45

act cag tcg ccc cag cag cct ggc tcc agg aac cgg ggg cgg ggc caa    315
Thr Gln Ser Pro Gln Gln Pro Gly Ser Arg Asn Arg Gly Arg Gly Gln
              50              55              60

ggg cgg ggc act gcc atg ccc ggg gag gag gtg ctg gag tcc agc caa    363
Gly Arg Gly Thr Ala Met Pro Gly Glu Glu Val Leu Glu Ser Ser Gln
          65              70              75

gag gcc ctg cat gtg acg gag cgc aaa tac ctg aag cga gac tgg tgc    411
Glu Ala Leu His Val Thr Glu Arg Lys Tyr Leu Lys Arg Asp Trp Cys
      80              85              90

aaa acc cag ccg ctt aag cag acc atc cac gag gaa ggc tgc aac agt    459
Lys Thr Gln Pro Leu Lys Gln Thr Ile His Glu Glu Gly Cys Asn Ser
95              100             105             110

cgc acc atc atc aac cgc ttc tgt tac ggc cag tgc aac tct ttc tac    507
Arg Thr Ile Ile Asn Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr
              115             120             125

atc ccc agg cac atc cgg aag gag gaa ggt tcc ttt cag tcc tgc tcc    555
Ile Pro Arg His Ile Arg Lys Glu Glu Gly Ser Phe Gln Ser Cys Ser
              130             135             140

ttc tgc aag ccc aag aaa ttc act acc atg atg gtc aca ctc aac tgc    603
Phe Cys Lys Pro Lys Lys Phe Thr Thr Met Met Val Thr Leu Asn Cys
          145             150             155

cct gaa cta cag cca cct acc aag aag aag aga gtc aca cgt gtg aag    651
Pro Glu Leu Gln Pro Pro Thr Lys Lys Lys Arg Val Thr Arg Val Lys
      160             165             170

cag tgt cgt tgc ata tcc atc gat ttg gat taa gccaaatcca ggtgcaccca  704
Gln Cys Arg Cys Ile Ser Ile Asp Leu Asp
175             180

gcatgtccta ggaatgcaga cccaggaagt cccagaccta aaacaaccag attcttactt  764

ggcttaaacc tagaggccag aagaaccccc agctgcctcc tggcaggagc ctgcttgtgc  824

gtagttcgtg tgcatgagtg tggatgggtg cctgtgggtg tttttagaca ccagagaaaa  884

cacagtctct gctagagagc acttcctatt ttgtaaacct atctgcttta atggggatgt  944

accagaaacc cacctcaccc cggctcacat ctaaaggggc ggggccgtgg tctggttctg  1004

actttgtgtt tttgtgccct cctggggacc agaatctcct ttcggaatga atgttcatgg  1064

aagaggctcc tctgagggca agagacctgt tttagtgctg cattcgacat ggaaaagtcc  1124

ttttaacctg tgcttgcatc ctcctttcct cctcctcctc acaatccatc tcttcttaag  1184

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aggtccagca aaagtcagta gggacattgc agaagcttga aaggccaata ccagaacaca   1424

ggctgatgct tctgagaaag tcttttccta gtatttaaca aaacccaagt gaacagagga   1484

gaaatgagat tgccagaaag tgattaactt tggccgttgc aatctgctca aacctaacac   1544

caaactgaaa acataaatac tgaccactcc tatgttcgga cccaagcaag ttagctaaac   1604

caaaccaact cctctgcttt gtccctcagg tggaaaagag aggtagttta gaactctctg   1664

cataggggtg ggaattaatc aaaaacctca gaggctgaaa ttcctaatac ctttccttta   1724

tcgtggttat agtcagctca tttccattcc actatttccc ataatgcttc tgagagccac   1784

taacttgatt gataaagatc ctgcctctgc tgagtgtacc tgacagtagt ctaagatgag   1844

agagtttagg gactactctg ttttaacaag aaatattttg ggggtctttt tgttttaact   1904

attgtcagga gattgggcta aagagaagac gacgagagta aggaaataaa gggaattgcc   1964

tctggctaga gagtagttag gtgttaatac ctggtagaga tgtaagggat atgacctccc   2024

tttctttatg tgctcacttg aggatctgag gggaccctgt taggagagca tagcatcatg   2084

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tgatctaagg gccaaagtgc agggtgggtg aactttattg tactttggat ttggttaacc   2264

tgttttcctc aagcctgagg ttttatatac aaactccctg aatactcttt ttgccttgtt   2324

acttctcagc ctcctagcca agtcctatgt aatatggaaa acaaacactg cagacttgag   2384

attcagttgc cgatcaaggc tctggcattc agagaaccct tgcaactcga gaagctgttt   2444

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cactactgat gattctcacg ctaggcgaat ttgtccaaac acatagtgtg tgtgtttgt    2624

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cagagtggat ttaattaagc acacaaatgc taaggcagaa ttttgagggt gggagagaag   2744

aaaagggaaa gaagctgaaa atgtaaaacc acaccaggga ggaaaaatga cattcagaac   2804

caccaaacac tgaatttctc ttgttgtttt aactctccca caagaatgca atttcgttaa   2864

tggagatgac ttaagttggc agcagtaatc ttctttagg agcttgtacc acagtcttgc   2924

acataagtgc agatttgccc caagtaaaga gaatttcctc aacactaact tcacggggat   2984

aatcaccacg taactaccct taaagcatat cactagccaa agagggaat atctgttctt   3044

cttactgtgc ctatattaag actagtacaa atgtggtgtg tcttccaact ttcattgaaa   3104
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            20   25   30

Ile Pro Pro Pro Asp Lys Ala Gln His Asn Asp Ser Glu Gln Thr Gln
            35   40   45

Ser Pro Gln Gln Pro Gly Ser Arg Asn Arg Gly Arg Gly Gln Gly Arg
        50   55   60

Gly Thr Ala Met Pro Gly Glu Glu Val Leu Glu Ser Ser Gln Glu Ala
65   70   75   80

Leu His Val Thr Glu Arg Lys Tyr Leu Lys Arg Asp Trp Cys Lys Thr
                85   90   95

Gln Pro Leu Lys Gln Thr Ile His Glu Glu Gly Cys Asn Ser Arg Thr
            100   105   110

Ile Ile Asn Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr Ile Pro
            115   120   125

Arg His Ile Arg Lys Glu Glu Gly Ser Phe Gln Ser Cys Ser Phe Cys
        130   135   140

Lys Pro Lys Lys Phe Thr Thr Met Met Val Thr Leu Asn Cys Pro Glu
145   150   155   160

Leu Gln Pro Pro Thr Lys Lys Lys Arg Val Thr Arg Val Lys Gln Cys
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Arg Cys Ile Ser Ile Asp Leu Asp
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<301>  Lah, M., Brodnicki, T., Maccarone, P., Nash, A., Stanley, E., and
       Harvey, R.P.
<302>  Human cerberus related gene CER1 maps to chromosome 9
<303>  Genomics
<304>  55
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<306>  364-366
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1               5               10              15


acc aca cgg cac cag gat ggc cgc cag aat cag agt tct ctt tcc ccc      96
Thr Thr Arg His Gln Asp Gly Arg Gln Asn Gln Ser Ser Leu Ser Pro
                20              25              30
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gta ctc ctg cca agg aat caa aga gag ctt ccc aca ggc aac cat gag        144
Val Leu Leu Pro Arg Asn Gln Arg Glu Leu Pro Thr Gly Asn His Glu
        35                      40                  45

gaa gct gag gag aag cca gat ctg ttt gtc gca gtg cca cac ctt gta        192
Glu Ala Glu Glu Lys Pro Asp Leu Phe Val Ala Val Pro His Leu Val
        50                      55                  60

gcc acc agc cct gca ggg gaa ggc cag agg cag aga gag aag atg ctg        240
Ala Thr Ser Pro Ala Gly Glu Gly Gln Arg Gln Arg Glu Lys Met Leu
65                  70                  75                  80

tcc aga ttt ggc agg ttc tgg aag aag cct gag aga gaa atg cat cca        288
Ser Arg Phe Gly Arg Phe Trp Lys Lys Pro Glu Arg Glu Met His Pro
                85                  90                  95

tcc agg gac tca gat agt gag ccc ttc cca cct ggg acc cag tcc ctc        336
Ser Arg Asp Ser Asp Ser Glu Pro Phe Pro Pro Gly Thr Gln Ser Leu
                100                 105                 110

atc cag ccg ata gat gga atg aaa atg gag aaa tct cct ctt cgg gaa        384
Ile Gln Pro Ile Asp Gly Met Lys Met Glu Lys Ser Pro Leu Arg Glu
                115                 120                 125

gaa gcc aag aaa ttc tgg cac cac ttc atg ttc aga aaa act ccg gct        432
Glu Ala Lys Lys Phe Trp His His Phe Met Phe Arg Lys Thr Pro Ala
        130                 135                 140

tct cag ggg gtc atc ttg ccc atc aaa agc cat gaa gta cat tgg gag        480
Ser Gln Gly Val Ile Leu Pro Ile Lys Ser His Glu Val His Trp Glu
145                 150                 155                 160

acc tgc agg aca gtg ccc ttc agc cag act ata acc cac gaa ggc tgt        528
Thr Cys Arg Thr Val Pro Phe Ser Gln Thr Ile Thr His Glu Gly Cys
                165                 170                 175

gaa aaa gta gtt gtt cag aac aac ctt tgc ttt ggg aaa tgc ggg tct        576
Glu Lys Val Val Val Gln Asn Asn Leu Cys Phe Gly Lys Cys Gly Ser
                180                 185                 190

gtt cat ttt cct gga gcc gcg cag cac tcc cat acc tcc tgc tct cac        624
Val His Phe Pro Gly Ala Ala Gln His Ser His Thr Ser Cys Ser His
        195                 200                 205

tgt ttg cct gcc aag ttc acc acg atg cac ttg cca ctg aac tgc act        672
Cys Leu Pro Ala Lys Phe Thr Thr Met His Leu Pro Leu Asn Cys Thr
        210                 215                 220

gaa ctt tcc tcc gtg atc aag gtg gtg atg ctg gtg gag gag tgc cag        720
Glu Leu Ser Ser Val Ile Lys Val Val Met Leu Val Glu Glu Cys Gln
225                 230                 235                 240

tgc aag gtg aag acg gag cat gaa gat gga cac atc cta cat gct ggc        768
Cys Lys Val Lys Thr Glu His Glu Asp Gly His Ile Leu His Ala Gly
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tcc cag gat tcc ttt atc cca gga gtt tca gct tga                        804
Ser Gln Asp Ser Phe Ile Pro Gly Val Ser Ala
                260                 265
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            20   25   30

Val Leu Leu Pro Arg Asn Gln Arg Glu Leu Pro Thr Gly Asn His Glu
        35   40   45

Glu Ala Glu Glu Lys Pro Asp Leu Phe Val Ala Val Pro His Leu Val
    50   55   60

Ala Thr Ser Pro Ala Gly Glu Gly Gln Arg Gln Arg Glu Lys Met Leu
65   70   75   80

Ser Arg Phe Gly Arg Phe Trp Lys Lys Pro Glu Arg Glu Met His Pro
            85   90   95

Ser Arg Asp Ser Asp Ser Glu Pro Phe Pro Pro Gly Thr Gln Ser Leu
            100   105   110

Ile Gln Pro Ile Asp Gly Met Lys Met Glu Lys Ser Pro Leu Arg Glu
        115   120   125

Glu Ala Lys Lys Phe Trp His His Phe Met Phe Arg Lys Thr Pro Ala
    130   135   140

Ser Gln Gly Val Ile Leu Pro Ile Lys Ser His Glu Val His Trp Glu
145   150   155   160

Thr Cys Arg Thr Val Pro Phe Ser Gln Thr Ile Thr His Glu Gly Cys
            165   170   175

Glu Lys Val Val Val Gln Asn Asn Leu Cys Phe Gly Lys Cys Gly Ser
            180   185   190

Val His Phe Pro Gly Ala Ala Gln His Ser His Thr Ser Cys Ser His
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Cys Leu Pro Ala Lys Phe Thr Thr Met His Leu Pro Leu Asn Cys Thr

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Glu Leu Ser Ser Val Ile Lys Val Val Met Leu Val Glu Glu Cys Gln
225   230   235   240

Cys Lys Val Lys Thr Glu His Glu Asp Gly His Ile Leu His Ala Gly
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Ser Gln Asp Ser Phe Ile Pro Gly Val Ser Ala
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<301>  ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M.,
       Miyazono, K., and Heldin, C.H.
<302>  Characterization of type I receptors for transforming growth
       factor-beta and activin
<303>  Science
<304>  264
<305>  5155
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<307>  1994
<308>  NM_001203
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<301>  Ide, H., Katoh, M., Sasaki, H., Yoshida, T., Aoki, K., Nawa, Y.,
       Osada, Y., Sugimura, T., and Terada, M.
<302>  Cloning of human bone morphogenetic protein type IB receptor
       (BMPR-IB) and its expression in prostate cancer in comparison
       with other BMPRs
<303>  Oncogene
<304>  14
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<306>  1377-1382
<307>  1997
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<300>
<301>  Ide, H., Saito-Ohara, P., Ohnami, S., Osada, Y., Ikeuchi, T.,
       Yoshida, T., and Terada, M.
<302>  Assignment of the BMPR1A and BMPR1B genes to human chromosome
       10q22.3 and 4q23-->q24 by in situ hybridization and radiation
       hybrid mapping
<303>  Cytogenet. Cell. Genet.
<304>  81
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<306>  285-286
<307>  1998
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<309> 2000-10-31
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<301> Astrom, A.K., Jin, D., Imamura, T., Roijer, E., Rosenzweig, B.,
      Miyazono, K., ten Dijke, P., and Stenman, G.
<302> Chromosomal localization of three human genes encoding bone
      morphogenetic protein receptors
<303> Mamm. Genome
<304> 10
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gtgaaaggaa aggaagatca tttcatgcct tgttgataaa ggttcagact tctgctgatt   180

cataaccatt tggctctgag ctatgacaag agaggaaaca aaaagttaaa cttacaagcc   240

tgccataagt gagaagcaaa cttccttgat aac atg ctt ttg cga agt gca gga   294
                                     Met Leu Leu Arg Ser Ala Gly
                                      1   5

aaa tta aat gtg ggc acc aag aaa gag gat ggt gag agt aca gcc ccc   342
Lys Leu Asn Val Gly Thr Lys Lys Glu Asp Gly Glu Ser Thr Ala Pro
        10   15   20

acc ccc cgt cca aag gtc ttg cgt tgt aaa tgc cac cac cat tgt cca   390
Thr Pro Arg Pro Lys Val Leu Arg Cys Lys Cys His His His Cys Pro
    25   30   35

gaa gac tca gtc aac aat att tgc agc aca gac gga tat tgt ttc acg   438
Glu Asp Ser Val Asn Asn Ile Cys Ser Thr Asp Gly Tyr Cys Phe Thr
40   45   50   55

atg ata gaa gag gat gac tct ggg ttg cct gtg gtc act tct ggt tgc   486
Met Ile Glu Glu Asp Asp Ser Gly Leu Pro Val Val Thr Ser Gly Cys
                60   65   70

cta gga cta gaa ggc tca gat ttt cag tgt cgg gac act ccc att cct   534
Leu Gly Leu Glu Gly Ser Asp Phe Gln Cys Arg Asp Thr Pro Ile Pro
            75   80   85

cat caa aga aga tca att gaa tgc tgc aca gaa agg aac gaa tgt aat   582
His Gln Arg Arg Ser Ile Glu Cys Cys Thr Glu Arg Asn Glu Cys Asn
            90   95   100

aaa gac cta cac cct aca ctg cct cca ttg aaa aac aga gat ttt gtt   630
Lys Asp Leu His Pro Thr Leu Pro Pro Leu Lys Asn Arg Asp Phe Val
    105   110   115

gat gga cct ata cac cac agg gct tta ctt ata tct gtg act gtc tgt   678
Asp Gly Pro Ile His His Arg Ala Leu Leu Ile Ser Val Thr Val Cys
120   125   130   135

agt ttg ctc ttg gtc ctt atc ata tta ttt tgt tac ttc cgg tat aaa   726
Ser Leu Leu Leu Val Leu Ile Ile Leu Phe Cys Tyr Phe Arg Tyr Lys
                140   145   150

aga caa gaa acc aga cct cga tac agc att ggg tta gaa cag gat gaa   774
Arg Gln Glu Thr Arg Pro Arg Tyr Ser Ile Gly Leu Glu Gln Asp Glu
                155   160   165

act tac att cct cct gga gaa tcc ctg aga gac tta att gag cag tct   822
Thr Tyr Ile Pro Pro Gly Glu Ser Leu Arg Asp Leu Ile Glu Gln Ser
            170   175   180

cag agc tca gga agt gga tca ggc ctc cct ctg ctg gtc caa agg act   870
Gln Ser Ser Gly Ser Gly Ser Gly Leu Pro Leu Leu Val Gln Arg Thr
            185   190   195

ata gct aag cag att cag atg gtg aaa cag att gga aaa ggt cgc tat   918
Ile Ala Lys Gln Ile Gln Met Val Lys Gln Ile Gly Lys Gly Arg Tyr
200   205   210   215

ggg gaa gtt tgg atg gga aag tgg cgt ggc gaa aag gta gct gtg aaa   966
Gly Glu Val Trp Met Gly Lys Trp Arg Gly Glu Lys Val Ala Val Lys
                220   225   230

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gtg ttc ttc acc aca gag gaa gcc agc tgg ttc aga gag aca gaa ata       1014
Val Phe Phe Thr Thr Glu Glu Ala Ser Trp Phe Arg Glu Thr Glu Ile
        235             240                 245

tat cag aca gtg ttg atg agg cat gaa aac att ttg ggt ttc att gct       1062
Tyr Gln Thr Val Leu Met Arg His Glu Asn Ile Leu Gly Phe Ile Ala
        250             255                 260

gca gat atc aaa ggg aca ggg tcc tgg acc cag ttg tac cta atc aca       1110
Ala Asp Ile Lys Gly Thr Gly Ser Trp Thr Gln Leu Tyr Leu Ile Thr
        265             270                 275

gac tat cat gaa aat ggt tcc ctt tat gat tat ctg aag tcc acc acc       1158
Asp Tyr His Glu Asn Gly Ser Leu Tyr Asp Tyr Leu Lys Ser Thr Thr
280             285                 290                 295

cta gac gct aaa tca atg ctg aag tta gcc tac tct tct gtc agt ggc       1206
Leu Asp Ala Lys Ser Met Leu Lys Leu Ala Tyr Ser Ser Val Ser Gly
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tta tgt cat tta cac aca gaa atc ttt agt act caa ggc aaa cca gca       1254
Leu Cys His Leu His Thr Glu Ile Phe Ser Thr Gln Gly Lys Pro Ala
            315             320                 325

att gcc cat cga gat ctg aaa agt aaa aac att ctg gtg aag aaa aat       1302
Ile Ala His Arg Asp Leu Lys Ser Lys Asn Ile Leu Val Lys Lys Asn
            330             335                 340

gga act tgc tgt att gct gac ctg ggc ctg gct gtt aaa ttt att agt       1350
Gly Thr Cys Cys Ile Ala Asp Leu Gly Leu Ala Val Lys Phe Ile Ser
    345             350                 355

gat aca aat gaa gtt gac ata cca cct aac act cga gtt ggc acc aaa       1398
Asp Thr Asn Glu Val Asp Ile Pro Pro Asn Thr Arg Val Gly Thr Lys
360             365                 370                 375

cgc tat atg cct cca gaa gtg ttg gac gag agc ttg aac aga aat cac       1446
Arg Tyr Met Pro Pro Glu Val Leu Asp Glu Ser Leu Asn Arg Asn His
            380             385                 390

ttc cag tct tac atc atg gct gac atg tat agt ttt ggc ctc atc ctt       1494
Phe Gln Ser Tyr Ile Met Ala Asp Met Tyr Ser Phe Gly Leu Ile Leu
            395             400                 405

tgg gag gtt gct agg aga tgt gta tca gga ggt ata gtg gaa gaa tac       1542
Trp Glu Val Ala Arg Arg Cys Val Ser Gly Gly Ile Val Glu Glu Tyr
            410             415                 420

cag ctt cct tat cat gac cta gtg ccc agt gac ccc tct tat gag gac       1590
Gln Leu Pro Tyr His Asp Leu Val Pro Ser Asp Pro Ser Tyr Glu Asp
        425             430                 435

atg agg gag att gtg tgc atc aag aag tta cgc ccc tca ttc cca aac       1638
Met Arg Glu Ile Val Cys Ile Lys Lys Leu Arg Pro Ser Phe Pro Asn
440             445             450                 455

cgg tgg agc agt gat gag tgt cta agg cag atg gga aaa ctc atg aca       1686
Arg Trp Ser Ser Asp Glu Cys Leu Arg Gln Met Gly Lys Leu Met Thr
                460             465                 470

gaa tgc tgg gct cac aat cct gca tca agg ctg aca gcc ctg cgg gtt       1734
Glu Cys Trp Ala His Asn Pro Ala Ser Arg Leu Thr Ala Leu Arg Val
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Glu Cys Trp Ala His Asn Pro Ala Ser Arg Leu Thr Ala Leu Arg Val
            475             480             485

aag aaa aca ctt gcc aaa atg tca gag tcc cag gac att aaa ctc tga     1782
Lys Lys Thr Leu Ala Lys Met Ser Glu Ser Gln Asp Ile Lys Leu
            490             495             500

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Lys Cys His His His Cys Pro Glu Asp Ser Val Asn Asn Ile Cys Ser
        35              40              45


Thr Asp Gly Tyr Cys Phe Thr Met Ile Glu Glu Asp Asp Ser Gly Leu
    50              55              60


Pro Val Val Thr Ser Gly Cys Leu Gly Leu Glu Gly Ser Asp Phe Gln
65              70              75              80


Cys Arg Asp Thr Pro Ile Pro His Gln Arg Arg Ser Ile Glu Cys Cys
                85              90              95


Thr Glu Arg Asn Glu Cys Asn Lys Asp Leu His Pro Thr Leu Pro Pro
                100             105             110


Leu Lys Asn Arg Asp Phe Val Asp Gly Pro Ile His His Arg Ala Leu
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Leu Ile Ser Val Thr Val Cys Ser Leu Leu Leu Val Leu Ile Ile Leu
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Phe Cys Tyr Phe Arg Tyr Lys Arg Gln Glu Thr Arg Pro Arg Tyr Ser
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Ile Gly Leu Glu Gln Asp Glu Thr Tyr Ile Pro Pro Gly Glu Ser Leu
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Arg Asp Leu Ile Glu Gln Ser Gln Ser Ser Gly Ser Gly Ser Gly Leu
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Pro Leu Leu Val Gln Arg Thr Ile Ala Lys Gln Ile Gln Met Val Lys
            195             200             205

Gln Ile Gly Lys Gly Arg Tyr Gly Glu Val Trp Met Gly Lys Trp Arg
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Gly Glu Lys Val Ala Val Lys Val Phe Phe Thr Thr Glu Glu Ala Ser
225             230             235             240

Trp Phe Arg Glu Thr Glu Ile Tyr Gln Thr Val Leu Met Arg His Glu
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Asn Ile Leu Gly Phe Ile Ala Ala Asp Ile Lys Gly Thr Gly Ser Trp
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Thr Gln Leu Tyr Leu Ile Thr Asp Tyr His Glu Asn Gly Ser Leu Tyr
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Asp Tyr Leu Lys Ser Thr Thr Leu Asp Ala Lys Ser Met Leu Lys Leu
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Ala Tyr Ser Ser Val Ser Gly Leu Cys His Leu His Thr Glu Ile Phe
305             310             315             320

Ser Thr Gln Gly Lys Pro Ala Ile Ala His Arg Asp Leu Lys Ser Lys
            325             330             335

Asn Ile Leu Val Lys Lys Asn Gly Thr Cys Cys Ile Ala Asp Leu Gly
            340             345             350

Leu Ala Val Lys Phe Ile Ser Asp Thr Asn Glu Val Asp Ile Pro Pro
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Asn Thr Arg Val Gly Thr Lys Arg Tyr Met Pro Pro Glu Val Leu Asp
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Glu Ser Leu Asn Arg Asn His Phe Gln Ser Tyr Ile Met Ala Asp Met
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385                    390                    395                    400
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Tyr Ser Phe Gly Leu Ile Leu Trp Glu Val Ala Arg Arg Cys Val Ser
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Gly Gly Ile Val Glu Glu Tyr Gln Leu Pro Tyr His Asp Leu Val Pro
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Ser Asp Pro Ser Tyr Glu Asp Met Arg Glu Ile Val Cys Ile Lys Lys
            435   440   445

Leu Arg Pro Ser Phe Pro Asn Arg Trp Ser Ser Asp Glu Cys Leu Arg
        450   455   460

Gln Met Gly Lys Leu Met Thr Glu Cys Trp Ala His Asn Pro Ala Ser
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Ser Gln Asp Ile Lys Leu
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